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Laccases from *Pleurotus ostreatus*: Production, Characterization and Application in Organic Solvent Systems with Special Respect to Polycyclic Aromatic Hydrocarbon Oxidation

by

Patrick Daniel Reimer

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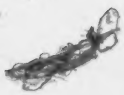

A Thesis

Submitted to the Faculty of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Master of Science

Department of Biological Sciences

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Laccases from *Pleurotus ostreatus*: Production, Characterization and Application in Organic Solvent Systems with Special Respect to Polycyclic Aromatic Hydrocarbon Oxidation submitted by Patrick Daniel Reimer in partial fulfillment of the requirements for the degree of Master of Science.

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Abstract

Several strains of white rot fungi were screened for their ability to produce laccase, a non-specific polyphenol oxidase. Of those tested, two strains were chosen for further study. The two strains chosen were *Pleurotus ostreatus* ATCC 58053 and UAMH 7988. A method for high laccase production was developed using a bran based medium; production for ATCC 58053 peaked at 15.6 U/mL and UAMH 7988 at 4.2 U/mL. Both laccases were extensively characterized and were similar in many respects to other fungal laccases. The two purified laccases are likely either very similar or identical to the earlier characterized pox2 laccase from *Pleurotus ostreatus* (strain Florida). The purified laccases were subjected to chemical modification to enhance their stability and activity in organic solvent systems. The polymers Brij35 and polyethylene glycol were attached to 34% and 45% of the available NH_2 groups of ATCC 58053 and UAMH 7988 laccases respectively. After modification, the enzymes retained the pH and temperature optima of the native forms of the enzyme. Modification did not alter substrate range but did slightly alter the kinetic constants of oxidation. After modification, the enzymes showed an increase in both their stability and activity in acetonitrile and dimethylsulfoxide, but not in N,N'-dimethylformamide. The modified enzymes displayed an enhanced ability to oxidize polycyclic aromatic hydrocarbons with an ionization potential below 7.5 eV in systems containing acetonitrile.

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1 Introduction

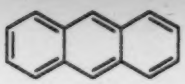
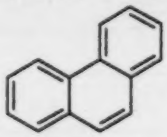
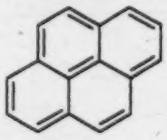
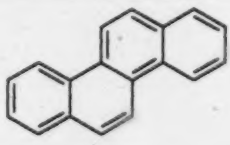
1.1 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are compounds composed of two or more fused benzene rings with or without hydrocarbon substituted groups or other linkages. The structures of these compounds can range from relatively simple to complex, but all share the aromatic character of the benzene rings that form their backbones. Table 1 demonstrates the structure and some important physiochemical properties of some representative PAHs. Members of this group of compounds often have toxic and carcinogenic properties (Keith and Telliard, 1979); this is a cause for significant and growing concern as several PAHs have become persistent environmental pollutants.

PAHs are typically found in creosote, petroleum, tars and sites contaminated with these products. PAHs also play roles in industrially important procedures and products; the active agent in mothballs is the PAH naphthalene. PAHs are also produced via incomplete combustion of carbon materials; they can be detected in wood smoke and vehicle emissions. In addition to fossil fuel combustion, another significant source of PAH exposure to the general population is through the inhalation of tobacco smoke (Lewtas, 1993).

PAHs are generally very hydrophobic and as a result have very low solubilities in aqueous systems. Bioaccumulation of these compounds is also a concern given their lipophilic nature. Most of the important characteristics of these compounds can be ascribed to their defining aromatic nature. The Π-electron charge stability and distribution of each PAH plays an important role in its susceptibility to the ease with which an electron can be removed during oxidation, typically to produce a cation radical. The relative ease of electron removal, or ionization potential, varies significantly among these compounds but does not

Table 1: Structure and properties of representative PAHs

PAH	Structure	Solubility (mM)	Ionization Potential (eV)
Anthracene		3.7×10^{-4}	7.55
Phenanthrene		7.2×10^{-3}	8.03
Chrysene		1.3×10^{-5}	7.80
Pyrene		7.2×10^{-4}	7.72

necessarily correlate with structural complexity. However, the positions of highest charge density within the compounds are usually most susceptible to nucleophilic substitution (Vazquez-Duhalt *et al.*, 1994). The ease with which these compounds are oxidized and their bioavailability play important roles in the extent of their biodegradation *in situ*. Bioavailability of PAHs is negatively affected by their strong absorption to soil matrices and low dissolution rate in contaminated environmental sites.

Due to their strong hydrophobic adsorption and low dissolution rate, PAHs are generally regarded as relatively persistent environmental pollutants. Much of the elimination of PAHs from such sites is a result of microbially mediated remediation, but other mechanisms of removal exist for these compounds. PAHs are also vulnerable to photo-oxidation, volatilization to the atmosphere, further adsorption to soil matrices, and other chemical oxidations to varying extents. Ecologically, PAHs tightly adsorbed to the soil matrix are less available for biodegradation and can present a difficult target for remediation.

The primary health concern associated with PAHs is their carcinogenicity. Benzo[a]pyrene was one of the first pure chemicals demonstrated to induce tumor formation in laboratory animals (Cook *et al.*, 1933; Hieger, 1933). Subsequent work has illustrated that PAHs are generally more precisely pericarcinogens as steps involved in mammalian metabolism of these compounds tend to produce detoxified products as well as proximate and ultimate carcinogens (Miller and Miller, 1985).

1.2 PAH Metabolism

Biodegradation of PAHs was studied as early as the 1920's (Gray and Thornton, 1928); however the most fruitful progress on elucidating the mechanisms involved has taken place in the last 25 years. As mentioned above, bioavailability is a concern for *in situ* degradation; further concerns include optimization of factors such as substrate

concentration, temperature, pH, per cent organic content and others that typically play a role in biological processes. Not surprisingly, biodegradation of lower molecular weight PAHs tends to proceed more rapidly than those of higher molecular weight. Metabolism of PAHs has been studied in several different organisms, however, bacterial and fungal systems have received the lion's share of attention both because of ease of study and the promise they hold for bioremediation.

1.2.1 Bacterial Systems

Most of the groundwork in the study of PAH metabolism was accomplished by examining bacterial systems. Several gram positive and gram negative bacteria have been isolated with the ability to metabolize PAHs and also use members of this class of compounds as sole carbon and energy sources (Cerniglia, 1993). The systems which bacteria use to degrade these compounds are intracellular; however, mechanisms of transport into the cell are still unclear but may involve non-specific transporters or diffusion for smaller molecular weight PAHs.

The enzymatic mechanisms of attack work similarly with most PAHs, not surprisingly given the similarities in structure seen with these compounds. Typically, the initial step in the process is dioxygenase mediated insertion of molecular oxygen to produce a cis-dihydrodiol intermediate. Dehydrogenases and other enzymes then produce intermediates suitable for ring cleavage. Once ring cleavage takes place, the products are typically broken down further to central metabolism intermediates. Figure 1 illustrates this process for naphthalene, the simplest PAH. The genetics governing naphthalene biodegradation in bacteria are well understood; however, such an understanding is not yet clear for all metabolized PAHs.

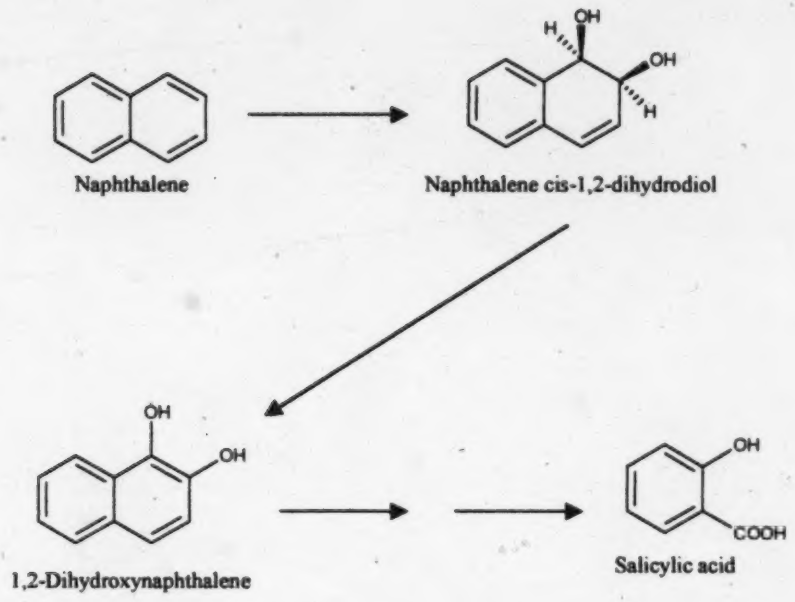


Figure 1: Steps typically involved in the bacterial metabolism of naphthalene

1.2.2 Fungal Systems

Several different fungi have also been noted for their ability to degrade PAHs, however, these systems in general have been less well studied than bacterial metabolism. In contrast to bacterial systems, fungi tend to transform PAHs as a result of co-metabolism, and often to dead-end metabolites (Andersson and Henrysson, 1996), although mineralization has been reported by numerous researchers (Bezalel *et al.*, 1996; Eggen and Majcherczyk, 1998; Field *et al.*, 1992). Significant detoxification of PAHs takes place as the metabolites formed are often less toxic or mutagenic than their parent compounds (Sutherland, 1992). Again, transport mechanisms for PAHs in fungal systems are poorly understood.

The key systems involved in these transformations involve intracellular cytochrome P_{450} monooxygenases (Cerniglia, 1993). Epoxidation of the PAH by this complex with molecular oxygen produces an unstable and highly reactive arene oxide. This arene oxide then as a rule either undergoes non-enzymatic rearrangement (to phenols or quinones) or is hydrated by an epoxide hydrolase to form trans-dihydrodiols. Both processes produce metabolites that may be subject to further transformations. These processes are illustrated with naphthalene in Figure 2.

1.2.3 Lignolytic Fungi

Secreted extracellular enzymes have also been demonstrated to play a role in PAH metabolism, primarily in lignin degrading white rot fungi (Bezalel *et al.*, 1996; Bogan and Lamar, 1995; Bogan *et al.*, 1996; Collins *et al.*, 1996; Field, *et al.*, 1992; Majcherczyk *et al.*, 1998; Milstein *et al.*, 1989; Sack *et al.*, 1997). Most work on fungal oxidation of PAHs has been performed using white rot fungi; in particular, *Phanerochaete chrysosporium*, and more recently, *Trametes versicolor*.

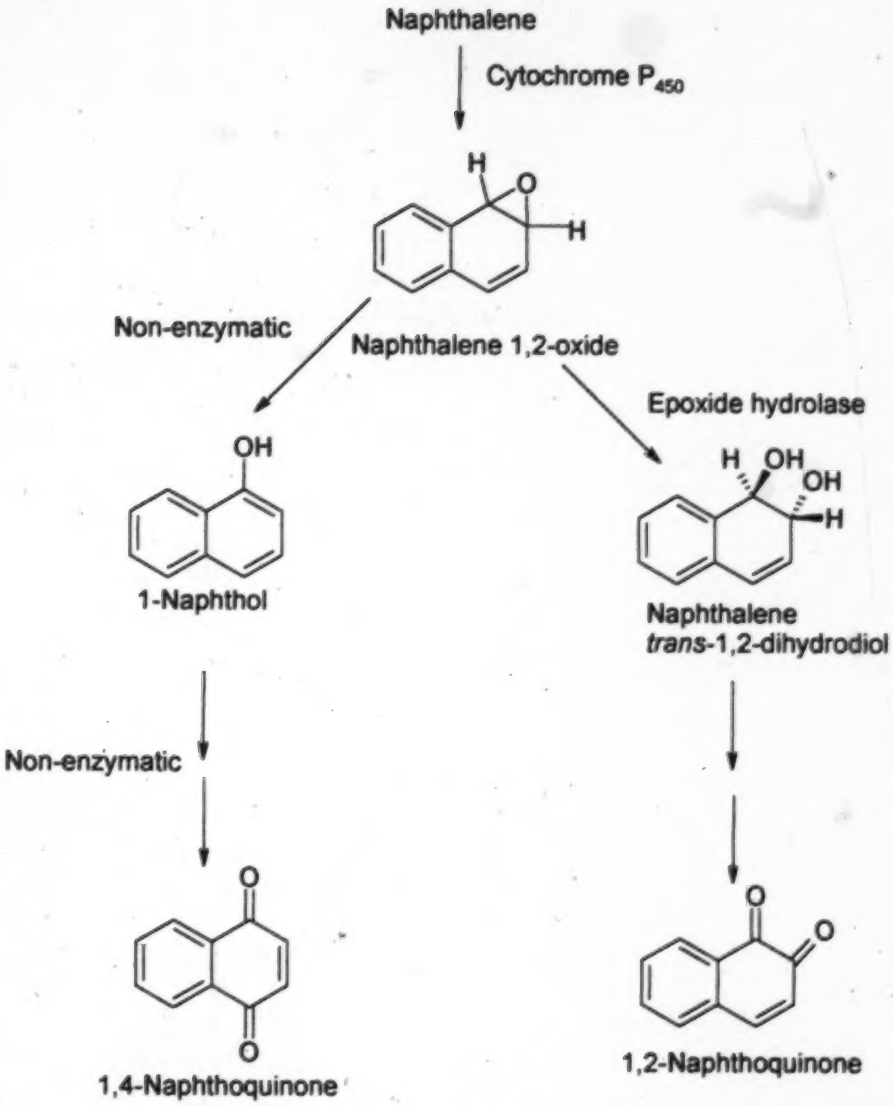


Figure 2: Preliminary steps involved in the fungal metabolism of naphthalene

Extracellular lignin peroxidases, laccases and manganese peroxidases catalyze one-electron oxidations of a wide variety of substrates in a non-specific manner, typically as part of lignin biodegradation. However, the broad substrate range of these enzymes and the structural similarities between lignin subunits and PAHs allows these enzymes to oxidize PAHs in a similar manner. After initial oxidation to produce a cationic radical PAH (typically unstable), the PAH is then subject to 1) further enzyme catalyzed oxidations (producing quinones) 2) non-enzymatic hydration and disproportionation or 3) polymerization with other cationic radicals. While these enzymes appear to play an important role in the initial steps in PAH metabolism by white rot fungi, *in vitro* assays demonstrate that they are incapable of mediating mineralization of PAHs alone (Bezalel *et al.*, 1997; Milstein, *et al.*, 1989). Further metabolism is likely mediated by other systems in these fungi, most likely the aforementioned cytochrome P_{450} route.

It is interesting to note that fungal mineralization of PAHs has only been observed using white rot fungi; the mechanism of metabolism clearly involves the use of extracellular ligninolytic enzymes but the details of the process remain unclear. In addition to facilitating mineralization, ligninolytic enzymes may also act to make PAHs more available to other organisms for biodegradation by producing more polar (and more water-soluble) metabolites. These transformed PAHs, in addition to being more bioavailable, are often less toxic and mutagenic (Sutherland, 1992).

1.3 White Rot Fungi

The best place to begin for a further understanding of white rot fungi is to look at the unique ecological niche that they occupy. White rot fungi are members of the division Eumycota, subdivision Basidiomycota, class Hymenomycetes, subclass Holobasidiomycetidae and order Aphyllophorales; this taxonomic designation contains fungi

involved in decompostion, and white rot fungi are the prime degraders of woody tissue (Burdsall, 1998). The primary ecological location for these saprobic basidiomycetes is dead, rotting wood. These organisms have several stages in their life cycle; the most important of which for nutrient acquisition and concomitant enzyme secretion is the long lived dikaryotic mycelium (hyphae) that grow in long and convoluted tracks throughout solid substrates. Under nutrient rich conditions, dikaryotic basidiocarp (fruiting bodies) are formed from these mycelia, which in turn produces haploid nuclei in basidia. These haploid nuclei then undergo karyogamy to produce diploid nuclei; these then undergo meiosis to form basidiospores, which then disperse, germinate and undergo plasmogamy to reform dikaryotic mycelium.

White rot fungi have been reported to successfully degrade a wide range of compounds, including PAHs, chlorinated aromatics (including polychlorinated biphenyls), pesticides, dyes, and munitions (including trinitrotoluene) (Barr and Aust, 1994). The non-specific and extracellular nature of the lignin degrading systems used by these fungi allow a wide range of chemicals to be attacked and at concentrations that would often be toxic to organisms using intracellular mechanisms alone. Further, these fungi can be cultivated using very inexpensive growth substrates containing woody or other plant tissue wastes. Several of these fungi are also readily cultivated in liquid media, allowing fermentation and large-scale production of extracellular enzymes.

Pleurotus ostreatus (commonly referred to as the oyster mushroom) is a white rot fungus that has received considerable attention. An important agricultural foodstuff, *P. ostreatus* has also been studied to a limited extent in relation to contaminant biodegradation (Bezalel, *et al.*, 1996; Reyes *et al.*, 1999) and extracellular enzyme production (Palmieri *et al.*, 1993; Sannia *et al.*, 1986).

Lignin is a complex structural polymer which provides both support and protection to plant tissue; wood is generally 20-30% lignin (Kirk and Farrell, 1987). Typically, lignin is found complexed with cellulose and hemi-cellulose; in fact, most white rot fungi do not utilize lignin as a primary energy source but instead use cellulose (Barr and Aust, 1994). Degradation of these polymers requires a broad set of enzymes because of the broad nature of the catabolism required; some of the more important enzymes are listed in Table 2. However, lignin biodegradation is a prerequisite for access to cellulose, and white rot fungi have several enzymes to accomplish this task. As can be seen in Figure 3, lignin is a very complex polymer consisting of non-repeating phenyl propanoid units linked by various carbon-carbon and ether bonds (Sarkanen and Ludwig, 1971). Figure 3 also illustrates some of the more common subunits of lignin, including sinapyl, coniferyl and p-coumaryl alcohol. Lignin is typically produced via a free radical based polymerization of these and other subunits. Lignin is rife with stereo-irregularity; further, chiral carbons exist in both D and L configurations. Given the structural complexity of lignin, it is easy to see why the mechanisms evolved to degrade it have had to be extracellular and non-specific.

1.4 Ligninolytic Enzymes

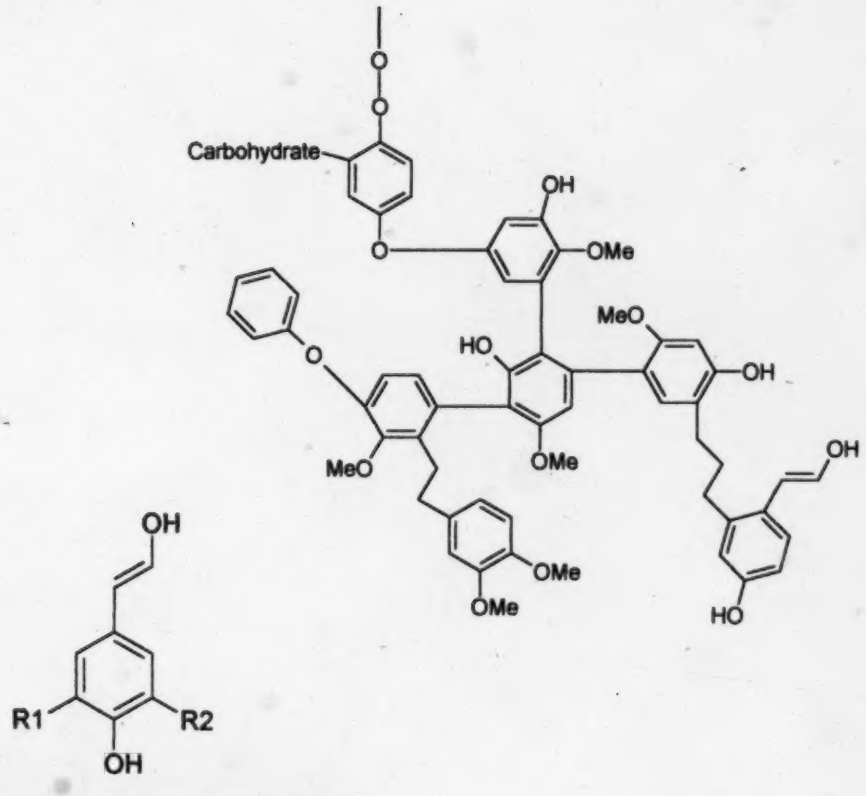
The enzymology of lignin degradation by white rot fungi is primarily governed by the action of three types of enzymes: lignin peroxidases, laccases and manganese peroxidases.

1.4.1 The Peroxidases

Lignin peroxidase (LiP) (EC 1.11.1.14) is a secreted, glycosylated enzyme typically between 38 to 43 kDa; manganese peroxidase (MnP) (EC 1.11.1.13) is also extracellular and glycosylated but is generally slightly larger at between 43 and 49 kDa (Heinzkill *et al.*, 1998). LiP has been isolated in a number of white rot fungi, including *Phlebia radiata*, *Panus tigrinus*, *Phanerochaete chrysosporium*, *Trametes versicolor*, and some strains of *Pleurotus ostreatus* and

Table 2: Enzymes involved in cellulose and hemi-cellulose degradation by white rot fungi

Substrate: cellulose	Substrate: hemi-cellulose
Endoglucanase	Endoxylanase
Cellobiohydrolase	Acetylxylan esterase
β-glucosidase	α-glucuronidase
	β-xylosidase
	Endomannase
	α-galactosidase
	Acetylglucomannan esterase
	β-mannosidase
	β-glucosidase



R1= R2= H : p-coumaryl alcohol
R1= OMe, R2= H : coniferyl alcohol
R1= R2= OMe : sinapyl alcohol

Figure 3: Schematic of the structure of lignin and common lignin subunits (adapted from (Kirk and Farrell, 1987))

Bjerkendera adusta (Kirk and Farrell, 1987); however, it is absent in a number of significant lignin degraders, including *Ceriporiopsis subvermispora* (Kirk and Cullen, 1998). MnP is also associated with a similar array of fungi, with notable producers including *C. subvermispora* and *B. adusta* (Kirk and Farrell, 1987).

Both of these enzymes catalyze one-electron oxidations of a broad range of substrates in a process consuming H_2O_2. These peroxidases contain heme in the form of protoporphyrin IX; at "rest", the iron is in the ferric state. H_2O_2 oxidizes the heme via the removal of two electrons from the ferric center, resulting in the production of the ferryl Π-porphyrin cation radical form of the enzyme, commonly called compound I (Barr and Aust, 1994). Compound I then interacts with and oxidizes a substrate by one electron with the concomitant reduction of compound I by one electron to compound II. Repetition of the single electron oxidation of a substrate returns the enzyme to its "resting" or ferric state, which is ready to be oxidized by H_2O_2 again. The basic steps of this process are illustrated in Figure 4. The heme center of LiP interacts directly with ultimate substrates; however, the oxidized heme of MnP interacts with Mn(II). Mn(II) is oxidized to Mn(III) via a single electron abstraction, then diffuses away and oxidizes substrates in turn.

The non-specific nature of these peroxidases allows them to oxidize a wide variety of substrates, including PAHs (Sutherland, 1992), dioxins, cyanides (Barr and Aust, 1994), and polychlorinated biphenyls (PCBs) (Beaudette *et al.*, 1998). These peroxidases have a considerably higher reduction potential than many others, including horseradish peroxidase (Millis *et al.*, 1989). This, coupled with the fact that specific active site conformational characteristics don't have to be met explains the range of substrates that can be oxidized. LiP has been used *in vitro* to oxidize PAHs with an ionization potential (IP) of up to 7.55 eV (measured by charge transfer) (Hammel *et al.*, 1986), although this value may not be an

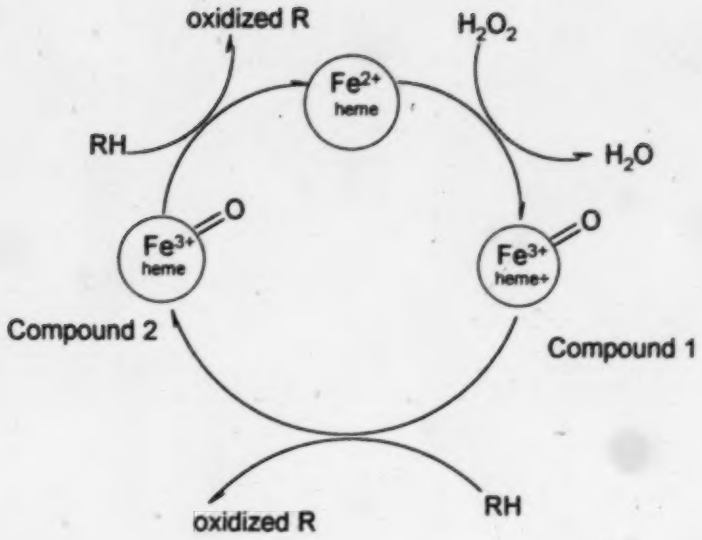


Figure 4: The mechanics of catalysis for lignin-degrading peroxidase enzymes from white rot fungi (adapted from (Barr and Aust, 1994))

absolute threshold as slightly different results have been obtained by other researchers using IP values obtained by electron impact (Vazquez-Duhalt, *et al.*, 1994). Early work demonstrated that MnP was similarly limited. However, more recent work has demonstrated that MnP may act on PAHs with higher IPs (ca. 8.0 eV) via a lipid peroxidation mechanism (Bohmer *et al.*, 1998).

1.4.2 Laccase

Laccase (EC 1.10.3.2), initially described by Yoshida (1883), has been extensively studied from a number of sources, including plants, fungi and bacteria; laccase types of activity have also been isolated from insect cuticles of a number of species (Gianfreda *et al.*, 1999). While plant laccases have been closely studied with respect to several important functions, contemporary work has centered on fungal laccases, and in particular, those from white rot fungi. Laccase has been isolated from a broad array of basidiomycetes, including *P. ostreatus*, *C. subvermispora*, *T. versicolor*, and *P. cinnabarinus* (Gianfreda *et al.*, 1999). Detailed information on the structure, mechanisms of action and functions of laccases from sources such as these has been collected.

1.4.2.1 Laccase Structure

Laccases are typically 60-80 kDa and can be monomeric, dimeric or tetrameric glycoproteins; carbohydrate contents are usually 15 to 20% by weight (Thurston, 1994). Sequencing of fungal laccase gene sequences has demonstrated that most are between 520 and 550 amino acids (including the requisite N-terminal secretion sequence). Most have acidic isoelectric points and demonstrate acidic pH optima for catalysis of a variety of substrates (Kirk and Farrell, 1987). Many fungi secrete more than one isoform of a given enzyme; this may be a result of separate genes but is more often an outcome of differential processing and/or glycosylation. Difficulties in obtaining a homogenous preparation may be

further compounded by heterogeneity introduced by proteolytic or glycosidic activities also typically produced in fungal media (Perry *et al.*, 1993).

1.4.2.2 The Active Site of Laccase and Catalysis

Laccases catalyze one-electron oxidations of a wide variety of substrates with the concomitant reduction of molecular oxygen to water. This is a relatively unique function; although many enzymes utilize molecular oxygen, only a specific few reduce it completely to water. Laccase shares this in common with ascorbate oxidase and ceruloplasmin (which are also called blue oxidases along with laccase); work on the crystal structure of this group of enzymes has yielded useful information for deductions about the active site of laccase (Call and Mucke, 1997).

Laccases and the other blue oxidases contain copper at their active site. Laccases contain four copper atoms, distributed as three types (types 1, 2 and 3). The single type 1 copper is responsible for the visible blue colour of purified preparations of this enzyme. In laccase, one cysteine and two histidines bind this copper; however, in other blue oxidases a fourth amino acid ligand, methionine, is often found. This difference may explain the more positive reduction-oxidation (redox) potential found in laccase at this center compared with other blue oxidases (Reinhammar and Malstrom, 1981). The two type 3 and one type 2 copper atoms are found in a trinuclear cluster slightly removed from the type 1 copper center. The type 2 and type 3 copper centers are nearly equidistant; the type 3 pair are 3.4 angstroms apart from each other and 3.9 A and 4.0 angstroms away from the type 2 copper. The type 3 copper atoms are bound by three histidine ligands; the remaining type 2 is bound by 2 histidine ligands (Ducros *et al.*, 1998). The cysteine and histidine residues that bind these copper centers and a small portion of their surrounding sequence are well conserved among the blue oxidases (Thurston, 1994).

Current thought on laccase catalysis divides the process into three steps; 1) the type 1 copper is reduced by the substrate being oxidized, followed by 2) transfer of the gained electron from the type 1 copper to the trinuclear cluster of type 2 and 3 coppers, and finally, 3) O_2 reduction to water at the trinuclear cluster (Gianfreda *et al.*, 1999). While single electron oxidations are being catalyzed, the reduction of molecular oxygen to water requires four electrons; the trinuclear cluster must act to store electrons in some way. Bound oxygen intermediates may also be involved but the complete process remains unclear (Messerschmidt *et al.*, 1992). However, H_2O_2 is not detected outside of laccase during steady state catalysis, demonstrating that a complete four-electron oxidation of molecular oxygen takes place. Although laccase is non-specific in terms of substrates to oxidize via single electron abstraction, it is very specific in only using molecular oxygen as the ultimate oxidizing agent; the O_2 binding pocket appears to restrict the access of oxidizing agents other than O_2 (Gianfreda *et al.*, 1999).

1.4.2.3 Laccase Substrate Range

Like the peroxidases of ligninolytic fungi, laccase has a wide substrate range; however, it is generally known for oxidation of aromatics containing an electron withdrawing group. Thus laccase is typically referred to as a polyphenol oxidase, and the classic reaction and its products are demonstrated in Figure 5. The free radical produced is generally unstable and subject to further enzyme catalysis (typically producing quinones), non-enzymatic hydration and disproportionation, polymerization with other cationic radicals, or internal rearrangement leading to bond cleavage (Marzullo *et al.*, 1995).

Accordingly, most laccase substrates have some aromatic character. However, some compounds such as $Fe(EDTA)^{2-}$ (Wherland *et al.*, 1975), ferrocyanide (Xu, 1996), ferrocytochrome c (Malmstrom *et al.*, 1971), aryl diamines and other inorganic ions

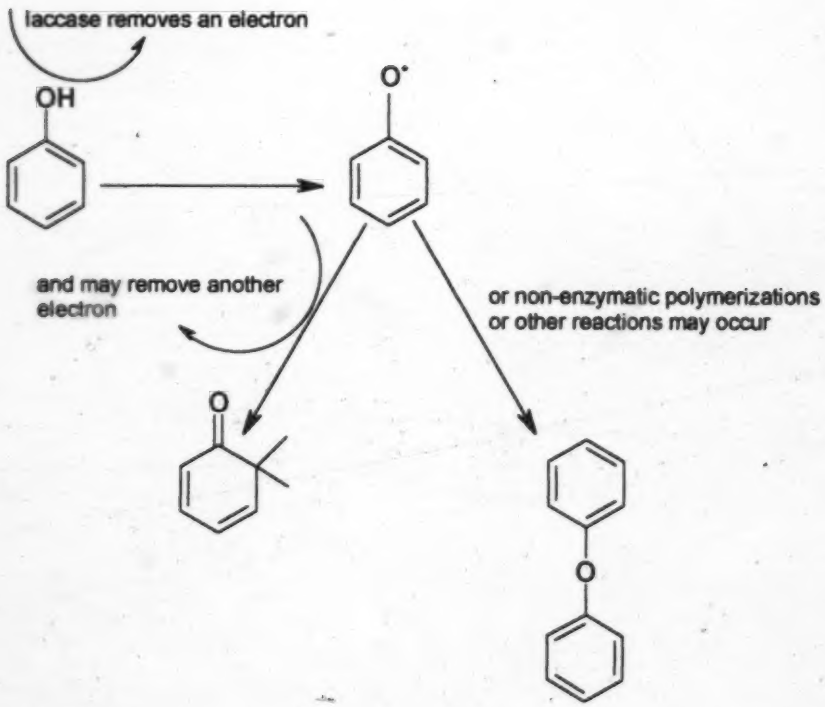


Figure 5: Typical examples of catalysis mediated by laccase

(Ghindilis *et al.*, 1992) without aromatic character have been reported as substrates. But the bulk of substrates analyzed and the best among them retain aromatic character and at least passing similarity to the lignin subunits demonstrated in Figure 3. Laccase can oxidize flavonoids (Pickard and Westlake, 1970), a variety of substituted monophenols, o-, m- and p-diphenols (Faure *et al.*, 1995), a variety of aromatic amines, 1-naphthol (Faure *et al.*, 1995), anilines, benzenethiols (Xu, 1996), 1-hydroxybenzotriazole (HBT) (Call and Mucke, 1997), phenoxazine (Schneider and Pedersen, 1995), syringaldezine, 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) and a bevy of similar compounds. However, laccase does not oxidize the aromatic amino acid tyrosine.

This broad substrate range must be made possible by enzyme-ligand binding that falls outside of the typical "lock-in-key" mechanism observed for many enzymes. Pickard and Westlake (1970) reported that no significant relationship between degree of hydroxylation or arrangement of hydroxyl groups on the flavonal nucleus and substrate specificity of *Trametes versicolor* laccase; further, the presence of sugar moieties at the C_3 position of a flavonoid (presumably significantly altering the shape of the substrate) resulted only in a decrease in the rate of reaction but not a change in overall substrate specificity. Later work by Xu (1996) utilizing methoxyphenols with p-substituents varying in size (up to 140 fold) yielded similar results. Results like these, coupled with recent information on the substrate pocket of *Coprinus cinereus* laccase (Ducros *et al.*, 1998) suggests that binding interactions are not significantly constrained as the substrate cleft in the enzyme may be relatively shallow.

Oxidation mediated by laccase does however appear to be significantly constrained in another respect. The redox potential of the type 1 copper site in fungal laccases is usually between 0.4 to 0.8 V (vs. normal hydrogen electrode) (Gianfreda *et al.*, 1999); a significant

variation. Munoz *et al.* (1997) demonstrated that laccase from *Pleurotus eryngii* was capable of oxidizing compounds with redox potentials of ≤ 0.6 V. In a more comprehensive examination, Xu (1996) demonstrated that the V_{max} rates of reaction with forty substrates were positively correlated with the differences in redox potential between the substrates and five enzymes tested. Although steric factors do not appear to play a large role in substrate specificity, the redox potential of a putative substrate does. In general, to be considered a potential substrate for laccase, most compounds would have to have a redox potential of < 1 V (Gianfreda *et al.*, 1999). Given the variation seen in fungal laccase redox potentials, significant variance in substrates oxidized (to the upper redox limit) could also be expected. Redox potentials for LiP and MnP have been observed as high as 1.49 V and 1.12 V respectively (Call and Mucke, 1997).

1.4.2.4 Laccase and Mediator Compounds

The range of compounds oxidized by laccase preparations may be increased to those of higher redox potential by the inclusion of small molecular weight "mediator" compounds. Sariaslani *et al.* (1984) noted the oxidation of rotenone by laccase when chlorpromazine was included in the reaction mixture; laccase alone was incapable of the oxidation. Later, Bourbonnais and Paice (1990) noted that in the presence of Remazol Blue and ABTS, laccase from *Trametes versicolor* could oxidize veratryl alcohol and non-phenolic lignin moieties whereas alone it could not. These early observations demonstrated the value of inclusion of mediator compounds in reaction systems; many further compounds have since been studied for mediating ability. Each successful mediator has been found to be a substrate of laccase (Call and Mucke, 1997). The most effective mediators to date are typically N-heterocyclics with N-OH groups; included in this cohort are ABTS and HBT.

While the exact mechanism of mediator action remains unclear, several theories have been put forth, based on experimental work using laccase systems with HBT and/or ABTS. Early theories included ideas that laccase was activated by the mediator via the donation of a single electron, active intermediates produced during oxidation of the mediator acted and were consumed, mediators acted in some way as a co-substrate, or active oxygen species produced during mediator oxidation then acted on the substrate (Li *et al.*, 1998). However, work by Bourbonnais *et al.* (1998) and Li *et al.* (1998) on the electrochemical nature of the reactions involved supports a separate hypothesis. The mediators are most likely oxidized to a nitroxy (HBT) or dication radical (ABTS) by laccase; these radicals in turn oxidize the substrates in question and are recycled back to their reduced form, to be oxidized by laccase again. The higher redox potentials of the nitroxy and dication radicals allow them to perform oxidations on a greater number of substrates than laccase would be able to oxidize alone.

The careful observer will note a seeming paradox in the above model; how could laccase oxidize mediators of a higher redox potential if it cannot oxidize other substrates with a higher redox potential? The answer may lie in the Nernst thermodynamic equation (Bourbonnais *et al.*, 1998). If the oxidation is driven forward by the reaction of the oxidized mediators with substrates, such a "step up" in redox potential is indeed mathematically possible. Laccase and mediator systems have been used to expand the range of PAHs (Johannes and Majcherczyk, 2000; Pickard *et al.*, 1999), industrial dyes (Reyes *et al.*, 1999), lignin and lignin derivatives (Bourbonnais and Paice, 1990; Bourbonnais *et al.*, 1995) oxidized. Further, industrial delignification systems for the pulp and paper industry have been designed (Call and Mucke, 1997).

Interestingly, mediator action with laccase may play a role in natural processes as well. An efficient lignin degrader, *Pycnoporus cinnabarinus*, has been found to produce a

compound which acts as a laccase mediator, 3-hydroxyanthranilate (Eggert *et al.*, 1996). This system could make sense in the natural context given the realtively large molecular weight of most fungal laccases (ca. 60 kDa), which would prevent them from entering the unaltered wood cell wall. Small molecular weight compounds could be oxidized by laccase, diffuse into the complex lignin structure and perform oxidations at sites inaccessible and redox potentials prohibitive to catalysis governed directly by the active site of the enzyme.

1.4.2.5 Laccase Applications

Like the other lignin degrading enzymes, laccase has been extensively examined for potential biotechnological applications. Again because of the broad substrate range of this enzyme, many avenues are possible; some of the most studied have included remediation of xenobiotic compounds. Laccase systems have been used to oxidize a wide and ever broadening array of important chemical pollutants. Some of the most relevant have included chlorinated and polychlorinated biphenyls (with dechlorination products) (Dec and Bollag, 1994; Roy-Arcand and Archibald, 1991), pesticides and their derivatives (Bollag and Liu, 1990), and various pollutants of wastewater from industrial processes, including several dyes (Chivukula and Renganathan, 1995). Many of the products from these reactions form polymeric aggregates of dimers, trimers or tetramers after reaction and formation of cationic radicals; it has also been demonstrated that the reactive intermediates may bind to natural humic constituents *in situ*. Inclusion of these compounds in the humification process in nature most likely plays a large role in their detoxification or availability for metabolism to other organisms (Gianfreda *et al.*, 1999).

Oxidation of PAHs has been studied as well, primarily over the last five years. Collins *et al.* (1996) utilized laccase from *Trametes versicolor* to oxidize two compounds with a relatively low IP, (≤ 7.45 eV) benzo[a]pyrene and anthracene. Majcherczyk *et al.* (1998)

Table 3: Polycyclic aromatic hydrocarbons oxidized by *Trametes versicolor* laccase Majcherczyk *et al.* (1998)

Acenaphthylene
Anthracene
Benzo[a]pyrene
Acenaphthene
Fluoranthene
Pyrene
Benzo[a]anthracene
Chrysene
Benzo[b]fluoranthene
Benzo[k]fluoranthene
Perylene

reacted *Trametes versicolor* laccase with the PAHs listed in Table 3. The products of oxidation included quinones and polymerized products, typical of laccase catalysis. While the best rates of oxidation were observed with compounds whose IP was below 7.5 eV, compounds with IPs of up to 7.8 eV were oxidized; further, no definite relationship between the oxidation of a compound and its ionization or redox potential could be gleaned. Bohmer *et al.* (1998) described the production of phenanthrene-9,10-quinone from phenanthrene with *Trametes hirsuta* laccase. Oxidation of a range of PAHs by *Coriolopsis gallica* laccase using ABTS and HBT mediator systems was noted by Pickard *et al.* (1999); the mass spectra of anthracene and acenaphthalene oxidation products revealed that the dione derivatives of these compounds were present. Recently, rates of oxidation of several PAHs with a number of different mediator compounds has been examined, demonstrating a significant variation depending on the mediator system used (Johannes and Majcherczyk, 2000).

Laccase has been studied for use in systems other than those designed for the remediation of xenobiotics. Laccase has been used in the production of indigo dye for textile dyeing; ironically, it also has several applications in textile bleaching as well (Damkus *et al.*, 1996). Laccase has been used for the oxidation of contaminating phenolics in wine (Kersten *et al.*, 1990) and in assays for the estimation of the phenolic content of fruit juices, tea and other beverages (Cliffe *et al.*, 1994). Measurement of α-glucosidase, α-amylase, cellobiose, quinone oxidase and glucose oxidase enzyme levels has also been performed in systems utilizing laccase. Gardiol *et al.* (1996) have also developed a sensitive gas phase biosensor using a plant laccase. Laccases have also been suggested as replacements for horseradish peroxidase (which can give high background staining) in enzyme-linked immunoassays; Bier *et al.* (1996) have also described the use of laccase in an extremely sensitive immunoassay.

Both the development of laccase applications and their implementation requires significant (several hundreds of milligrams) production of the enzyme; several strategies exist for enhancing production, as discussed below. Current industrial producers of laccase include Novo Nordisk A/S, Juelick Enzyme Products, and Tienzyme, Inc. Currently, most of the laccase production of these companies is directed towards sales in the pulp and paper industry.

1.5 Laccase Production

The optimization of production of many industrially important enzymes, including laccase, has been the focus of much study in the 20th century. Valuable progress has been made in increasing production titre by optimizing media and growth conditions, screening of strains for improved production qualities, and mutagenesis of selected strains for increased production. A valuable addition to this toolbox has been the use of genetic engineering to produce recombinant laccase. The uptake of this technology by industry has been rapid over the last decade. For example, in 1991 only a small fraction of the enzyme production by Novo Nordisk A/S was manufactured on the basis of genetic engineering; by 1996 over one half of the enzyme products of this company were produced by these methods.

1.5.1 Recombinant Production

The complete sequences of over thirty laccase genes have now been cloned, satisfying the first and most obvious prerequisite for recombinant production (Gianfreda *et al.*, 1999). Several of these genes have been used to attempt recombinant production by both heterologous and homologous means (Kojima *et al.*, 1990). Laccase, like most fungal proteins, is highly processed; a further complication is that it must be successfully secreted to the production medium. Potential hosts should then be capable of secreting fungal proteins in large amounts, and be simple to cultivate with good growth characteristics and genetic

stability. Two of the more successful hosts used to date have been *Aspergillus oryzae* and *Trichoderma reesei*. These fungi have been used successfully in the production of several other commercially important enzymes as well. Given the complexities of fungal growth characteristics and difficulties involved in constructing functional vectors (with favourable promoters, terminators, the correct gene sequence, transcription and other factors) and their insertion, designing a viable production system via these methods can be arduous and time consuming. However, increases in yields can be dramatic once conditions are optimized. Yields of laccase in recombinant systems have been reported as high as 20 mg/L in shake flask experiments (Berka *et al.*, 1997). However, it is reasonable to assume that optimized proprietary industrial fermentation methods achieve significantly greater yields. It will also be interesting to see if the recent backlash against genetically modified products spreads to enzyme production by recombinant means.

Despite the recent advance of recombinant technology for laccase production, most work to date on the subject has centred on screening for high producing wild-type isolates or the optimization of growth conditions, including media design. While many researchers have examined a small number of isolates for laccase producing ability, a few large-scale screening efforts have also been carried out.

1.5.2 Isolate Screening

Well-designed screening studies for basidiomycete laccase production must deal with several constraints that are primarily a function of the nature of fungal growth. Growth of fungi is often much more complex than that of the typical sigmoidal curve of bacterial growth kinetics and can depend on several factors in the medium and their time of appearance. Further, fungal growth dynamics often take place over a period of days, instead of the hours seen in bacterial systems. Fungal extracellular enzyme production is also

regulated in a complex and not fully understood manner. However, several reports indicate that this enzyme production is a function of secondary metabolism; these energy-harvesting enzymes are produced when primary (e.g. simple sugars) energy sources are depleted.

Several researchers have screened basidiomycetes for rate of growth and lignin degradation or dye decolourization (Esposito *et al.*, 1991), however, few have attempted to correlate enzyme production with these criteria or directly measure enzyme production alone. Of those that have, Rodriguez *et al.* (1998) and de Jong *et al.* (1992) correlated enzyme activity in liquid growth media with the primary screening technique of dye oxidation and decolourization. Rodriguez *et al.* (1998) found a significant correlation between dye decolourization and laccase production across several strains of white rot fungi. Pelaez *et al.* (1995) screened 68 basidiomycetes for laccase, MnP and aryl-alcohol oxidase production in a defined, glucose based liquid media. White rot fungi were found to perform well against other fungi tested, but overall enzyme production was low compared with values obtained in other studies. Defined, sugar rich media like that used in the studies above tend to be poor at supporting the production of these enzymes, because of their association with secondary metabolism. A method of particular use for screening large numbers of fungi for laccase production appears to be dye decolourization. Several suitable dyes are easily incorporated into solid or liquid media, are inexpensive, stable, have a low toxicity and remain extracellular (de Jong *et al.*, 1992).

1.5.3 Inducers

Early in the study of laccase, it was noted that several compounds that do not necessarily act as substrates of the enzyme could nonetheless induce its synthesis when present in the growth media (Pickard and Westlake, 1970). Several researchers have examined a number of compounds, typically small phenolics, as can be seen in Table 4. Of

Table 4: Common compounds used to induce laccase production during fungal fermentations

p-Anisidine
Benzyl Alcohol
Ferulic acid
Orcinol
p-Toluidine
2,5-Xylidine
Veratryl alcohol

these compounds, 2,5-xylidine has produced the best and most consistent results across a number of strains (Bollag and Leonowicz, 1984). The mechanism of action of these inducers remains unstudied; however, the similarity between the most successful inducers and the structural subunits of lignin cannot be overlooked. Presumably, these small molecular weight compounds diffuse into hyphal cells and either transcriptionally or translationally enhance laccase production and secretion. Collins and Dobson (1997) demonstrated that amounts of laccase mRNA and activity were a direct function of the concentration of 2,5-xylidine or another inducer, HBT in cultures of *T. versicolor*. Increased transcript levels in this study were noted within 15 minutes of the addition of 2,5-xylidine.

Other less obvious substances have been used to induce laccase production as well. Chief among them is copper, an important component of the active site of laccase. Recent work has demonstrated its value as an inducer (Collins and Dobson, 1997; Palmieri *et al.*, 2000) in some strains, whereas it has failed to have an effect in others (Fukushima and Kirk, 1995). Support for the idea that copper can be the limiting factor in active laccase production has been found in studies of plant production of the enzyme (Gianfreda *et al.*, 1999). Cycloheximide has also been observed to stimulate the production of laccase, but it is difficult to speculate as to the mechanism of action (Grotewold *et al.*, 1988). Dombrovskaya and Kostyshin (1996) reported on the use of surfactants to slightly increase laccase production. However, only the apparent activity of laccase may have been increased as 1) residual surfactant may have increased the activity of laccase via a lipid-peroxidation mechanism, similar to that proposed by Bohmer *et al.* (1998) or 2) residual surfactant may also aid in solubilization of substrate, as seen by Boonchan *et al.* (1998), resulting in stronger responses in the enzyme assay.

Concentration of the added inducer also plays an important role; because several of the effective small phenolics can be toxic at higher concentrations, the optimum level often has to be determined empirically. Rogalski *et al.* (1991) observed this threshold effect with xylidine induction of *Phlebia radiata* and *P. cinnabarinus* cultures. Timing of the addition of the inducer chosen also has an effect on the final increases in laccase titre seen (Shuttleworth *et al.*, 1986). Typically, best results are seen with the addition taking place after the initial, rapid increase in biomass in the early phases of growth in liquid culture.

1.5.4 Lignin Based Media

Considerable effort has also been directed towards optimizing media for laccase production as well. As mentioned, defined media tend to be relatively poor; this, coupled with the plethora of cheap and available lignin sources has supported the investigation of a number of alternatives. Table 5 demonstrates most of the published attempts to date related to increasing laccase production via the use of lignin based substrates. All have been successful to a degree, ranging from a doubling of production (Vasdev and Kulad, 1994) to a 10-fold increase (Pickard *et al.*, 1999). The use of these sugar poor, lignin rich media presumably shifts the fungi to secondary metabolism both earlier and more vigorously; in most cases a greater peak production of laccase is also seen earlier. It is reasonable to assume that the entire ligninolytic machinery is activated (perhaps via induction from small molecular weight breakdown products of lignin) in these cases, and laccase levels increase concomitantly.

1.5.5 Strain Mutation

A traditional method of increasing enzyme or other biomolecule production has been centered on the creation of mutants with enhanced ability with respect to the compound of interest. Some limited work has been performed in attempts to increase

Table 5: Studies on laccase production using lignin-based media

Organism	Medium	Reference
T. versicolor	Wheat straw, beech wood	(Schlosser *et al.*, 1997)
P. ostreatus	Cotton stalk extract	(Ardon *et al.*, 1998)
T. versicolor, P. chrysosporium	Whole oats	(Rodriguez *et al.*, 1998)
Coriolopsis. gallica	Cereal bran	(Pickard *et al.*, 1999)
Flammulina velutipes	Barley straw	(Lee and Suh, 1985)
Pleurotus pulmonarius	Cotton wheat straw	(Masaphy and Levanon, 1992)
Polyporus brumalis	Potato wastes	(Trojanowski *et al.*, 1995)
Lentinus edodes	Corn straw and chestnut juice	(Crestini *et al.*, 1996)
Ceriporiopsis subvermispora	Wheat bran	(Salas *et al.*, 1995)
Pycnoporus cinnabarinus	Kraft lignin	(Gomez-Alarcon *et al.*, 1989)
Cyathus bulleri	Kraft lignin	(Vasdev and Kulad, 1994)
Pleurotus florida	Paddy straw	(Dhaliwal *et al.*, 1992)

laccase production via mutagenization and screening of white rot fungi. Dhaliwal *et al.* (1992) created *Pleurotus floridae* protoplasts and mutagenized them with N-methyl-N'-nitro-N-nitrosoguanidine; one resultant strain showed a 128% increase in laccase production in a glucose based medium and a 52% increase in a paddy straw based medium over the parent. Homolka and Nerud (1995) used UV mutagenization of *P. ostreatus* protoplasts to produce strains with a mean increase in laccase production of 38%. The increases in production generated by these experiments are meaningful, however, optimization of media has been historically more successful in increasing yields.

1.5.6 Fungal Fermentations and Laccase Production

A survey of the methods used by various researchers for the production of laccase uncovers the fact that no one protocol is widely followed. This is a function of both the idiosyncrasies of fungal enzyme production and the various laboratories involved. However, in the production of laccase, as in other fungal fermentations, several basic prerequisites for success exist.

Typically, design of a fungal fermentation system begins with the use of shake flasks. Dozens of shake flasks can be accommodated on a single shaking platform and this allows the investigation of several media or other growth conditions in statistically significant numbers. Inoculum preparation for these experiments typically involves the excision of fungal material from an agar plate and its homogenization in a defined medium. Subsequent growth in the defined medium is rapid, and biomass quickly increases. At the peak of growth, this culture is again homogenized and fractions are used to inoculate the media being tested. Maintenance of contamination free stocks for inoculation is of obvious importance. Several researchers have reported difficulty with contamination from mites, bacteria, and other fungi. Eggert *et al.* (1996) grew *P. cinnabarinus* on agar plates containing

lignosulfate before their use in inoculation; such a preliminary step allows for the early induction of lignolytic enzymes. During shake flask experiments, valuable information about the utility of different media compositions and general growth dynamics of the fungus can be learned.

Some of the important parameters governing the success of a fungal fermentation include dissolved oxygen concentration, medium pH, culture viscosity and mixing, and temperature. In addition to offering larger scale production, use of commercial fermenters allows closer control of these factors via defined oxygen or air introduction, acid & base addition, impeller design and speed, and internal heating & cooling systems. Significant increases in production have been noted on scale-up to fermenters from shake flask experiments; Eggert *et al.* (1996) noted a twofold increase in production of laccase on scale up to a 100 L fermenter. Typically, stirred tank fermenters are employed for most large-scale fungal fermentations; successful and reproducible runs have been carried out with volumes as large as 200 000 L (Jones, 1998). Inoculation of fermenters generally follows the same pattern as that for shake flasks. However, additional growth stages in larger volumes of defined media are usually incorporated to produce the relatively larger amount of biomass required.

Important for enzyme production in several species is the morphology of the fungal material in culture. Fungi in liquid culture tend to grow in either a hyphal or pelleted form. Hyphal growth, while necessary in some species for production, creates a more viscous culture and one that is more sensitive to shear stress from impeller motion. Many fungi exhibit pelleted growth in liquid media. Pellet size and composition can be important; if pellets grow too large, then serious mass transfer limitations between the interior of the

pellet and the surrounding medium can exist. Fine control of fermentation parameters can be required to ensure that the desired fungal morphology is seen.

1.6 Enzymes and Organic Solvents

Many potential substrates of laccase oxidation, including PAHs, are only sparingly soluble in water. Therefore, use of this enzyme in aqueous systems for the oxidation of PAHs would be severely limited by mass transfer considerations of the substrate. PAHs are several times more soluble in organic solvents than water; a reaction system utilizing such solvents could conceivably address such insolubility and mass transfer concerns.

Enzyme catalysis in organic solvents has received considerable attention since the early 1900's (Klibanov, 1989), primarily because of the increased possibilities in these environments, especially for industrial processes. Organic solvents can provide advantages in increased ease of product recovery, shifting of thermodynamic equilibria, enhanced substrate solubility, reduced microbial contamination and other areas (Khmelnitsky *et al.*, 1988). Work in the field has expanded rapidly in the last twenty years however, and this recent expansion can trace its roots to work in the mid 1980s by Klibanov and collegues, who reported significant bioconversions in nearly anhydrous solvents (Brink *et al.*, 1988). Much work has been performed on serine proteases and lipases, and applications involving these and other enzymes have been developed. Coincident with these advances has been the study of the effect of such solvent environments on enzyme activity; significant progress has been made in elucidating important underlying mechanisms.

1.6.1 Enzyme-Solvent Interactions

The native environments of enzymes are aqueous; it is reasonable to assume that their catalytic abilities have been optimized over millennia for this environment. To date, no

enzyme has been noted to function in a truly anhydrous solvent; however, the volume of water required in some systems has been calculated as being less than that necessary to form a monolayer over the enzyme (Garza-Ramos *et al.*, 1992). Considerable flexibility is afforded in the choice of solvent for different applications, especially in terms of solvent hydrophobicity and miscibility with water. Although some enzymes have been noted to retain significant amounts of activity in hydrophobic solvents, these systems tend to have difficulties that can preclude detailed analysis of the reactions catalyzed therein. Hydrophobic solvents often require the addition of enzyme as a powder or in an immobilized form because of solubility concerns; this may play havoc with detailed kinetic measurements, as substrate transfer and product removal in these heterogeneous systems may affect the results in a way not seen with a completely solubilized catalyst.

Water plays an important role in the structure and activity of enzymes. Typically, several sites on the surface of an enzyme are bound by water, and internal sites of solvation may exist as well (Gregory, 1995). As such, these molecules of water play important roles in determining the web of electrostatic interactions that influence the final conformation of the protein (Khmelnitsky *et al.*, 1988). When non-aqueous solvents are utilized, if they are more hydrophilic than the surface of the protein, it will be energetically more favourable for water to enter the solvent. This process will also be driven by the increase in entropy resulting from the release of bound water. If the free energy of this process overcomes the activation energy of the resultant unfolding and reorganization of the protein, denaturation of the enzyme will be the result. Further, loss of a "shell" of hydration around the enzyme will increase the vulnerability of the enzyme to electrostatic effects from the solvent, leading to further alterations in structure at critical hydrogen bonding and other sites (Russel *et al.*, 1992). Beyond effects on conformation, hydration of an enzyme can also play a role in the

dynamic nature of enzymatic catalysis and "flexibility". Water molecules can be important in the transient alterations in shape that play an important role in the catalytic cycles of several enzymes (Russel *et al.*, 1992).

Fully dehydrated enzymes are typically inactive; it has been demonstrated that incremental increases in the hydration of lysozyme and other enzymes produces a sigmoidal curve describing increased activity, to a plateau of catalysis which usually is reached before the enzyme is fully hydrated (Finney and Poole, 1984). Saskia van Erp and colleagues (1991) have described this S-shaped dependence of V_{max} on hydration by the following Hill-type equation:

$$V_m = V_{mhydr}\, w_p^{\,n} \;/\; K_1 + w_p^{\,n}$$

Where V_m is the maximal velocity at a given enzyme hydration degree, V_{mhydr} is the maximal velocity at full hydration, w_p is the hydration degree of the enzyme and K_1 and n are constants.

The addition of increasing concentrations of water-miscible organic solvents produces similar sigmoidal curves in initial enzyme activity, as noted by several researchers (Khmelnitsky *et al.*, 1991; van Erp *et al.*, 1991; Vazquez-Duhalt *et al.*, 1993). Presumably, the addition of these solvents displaces water from the enzymes tested, and the loss in activity is a result of dehydration. At this point, it is important to note that studies of this type should be performed by looking at complete V_{max} and K_m values. Many studies have fixed substrate concentration and altered water content or the nature of the organic solvent to determine the effects on enzyme catalysis. However, this provides only a snapshot of activity and is a relatively one-dimensional view; the paucity of data supplied by this approach prevents significant comments on the overall catalytic abilities of the enzymes in these systems from being made. Numerous researchers have noted alterations in K_m in the orders of magnitude

range upon the addition of organic solvents, even in relatively low concentrations (van Erp *et al.*, 1991; Vazquez-Duhalt *et al.*, 1992; Vazquez-Duhalt *et al.*, 1994). At fixed substrate concentrations, these alterations in K_m may lead to erroneous conclusions about maximal activity in these systems.

As previously mentioned, addition of increasing amounts of water-miscible organic solvents creates a "threshold effect" on the catalytic ability of most enzymes as the protein is dehydrated and denatured. An example of this is presented in Figure 6. Typically, the concentration of organic solvent resulting in a 50% reduction in initial enzyme activity is called the threshold concentration, or EC_{50} of the solvent. Several researchers have attempted to correlate physiochemical parameters of solvents with this decrease in enzyme activity; generally poor results have been obtained when trying to correlate to any one factor, including the traditional measure of hydrophobicity, the partition coefficient of a solvent between octanol and water phases. Khmelnitsky *et al.* (1991) proposed the following relationship correlating the threshold concentration of a solvent with its hydrophobicity:

$$\log W_{50}/C_{50}^{n} = B_0 + B_1 n + B_2 E_T(30) + B_3 n \log P$$

Where W_{50} is the concentration of water in the system at which half-inactivation occurs, n is the ratio of molecular surface area of water to that of the organic solvent, E_T (30) is the Dimroth-Reichardt solvent polarity parameter, P is the partition coefficient of the solvent in an octanol:water system, and the coefficients B_x are constants for a given protein. However, other researchers have found a poor fit with this model. Torres *et al.* (1996) proposed another model, also ultimately aimed at predicting EC_{50} from calculable parameters of solvents. This model holds that the important aspects of hydrophobicity could be governed by the following equation:

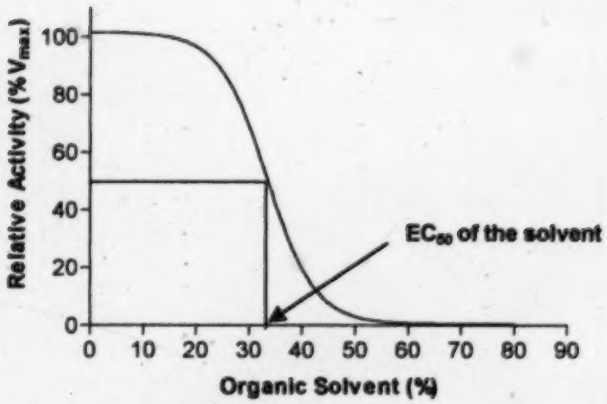


Figure 6: Generalized catalytic ability of an enzyme at increasing concentrations of a water-miscible organic solvent.

$H = a_s/E_T(30)$

Where H is the hydrophobicity parameter and a_s is the thermodynamic activity of the organic solvent in the solvent:water mixture.

These researchers found a linear correlation between the hydrophobicity parameter value of the solvent at EC_{50} (H_{50}) and the E_T (30) of the solvent. This relationship could allow the calculation of EC_{50} for a given solvent with an enzyme for which this linear realtionship had already been determined. Although these researchers have reported a good fit using LiP and cytochrome c, this model has yet to be rigorously tested.

1.6.2 Enzyme Modifications for Improved Activity in Organic Solvents

Researchers have also attempted to modify enzymes in various ways to prevent denaturation due to dehydration in organic solvents. Key among these methods are immobilization, cross-linking, protein engineering, and chemical modification.

1.6.2.1 Immobilization

In attempts to ensure enzymes retain conformation in dehydrating organic solvents, several researchers have immobilized the proteins on various supports. Enzyme preparations are typically dried onto the supports, which are then included in the reaction mixtures to be tested. It is hypothesized that binding to these supports aids enzymes in retaining their conformation and activity by locking in at least a portion of their structure in place. Covalent binding of this nature of enzymes to various supports has provided stability against fluctuations in ionic concentrations and pH, increased half life, and improved activity in organic solvents (Ruiz *et al.*, 2000). Laccase has been immobilized and used in systems of effluent decolourization (Davis and Burns, 1992), studies on the effects of organic solvent (van Erp *et al.*, 1991), and in PAH oxidation (Milstein *et al.*, 1989).

1.6.2.2 Cross-Linking

Another method for increasing stability is the use of bi- or poly functional reagents to bind with enzymes and create a matrix with inter or intramolecular bonds. A commonly used crosslinking agent is glutaraldehyde; it binds to and links amino groups of lysines on different enzyme moieties (DeSantis and Jones, 1999). In addition, enzyme microcrystals can be linked as well. Several researchers studying crosslinked enzyme crystals (CLECs) have found that they are highly active and stable in organic solvents (Govardhan, 1999). However, problems with mass transfer of substrates within the relatively large (ca. 10^{-1} mm in diameter) microcrystal arrays can be rate limiting if the process and product are not fully optimized.

1.6.2.3 Protein Engineering

Another avenue for increasing activity in organic solvents involves the engineering of the amino acid sequence for greater stability. Substantial improvements have been noted with minor alterations in sequence; extensive work by Frances Arnold and colleagues has produced a rough set of guidelines for engineering stability in non-aqueous solvents (Arnold, 1990). These design considerations are aimed at increasing conformational stability by maximizing internal crosslinks, van der Waals interactions, hydrogen bonds, and interior electrostatic interactions. In addition, alterations in amino acids at the surface of a protein can be made to increase compatability with solvent systems. Recent advances in directed evolution of proteins (Schmidt-Dannert and Arnold, 1999) may expand the realm of protein engineering for activity in organic solvents further.

1.6.2.4 Chemical Modifications

Another method of increasing protein stability in organic solvents involves the covalent attachment of polymeric functionalities such as polyethylene glycol (PEG). Attachment of these polymeric groups is often achieved via their activation with triazine ring derivatives (e.g. cyanuric chloride) the triazine ring then binds to surface amino groups on

the protein under alkali conditions, linking the polymer to the surface of the enzyme. Several groups have reported success in increasing the stability of enzymes with these techniques (Herniaz *et al.*, 1999; Koops *et al.*, 1999; Secundo *et al.*, 1999; Siddiqui *et al.*, 1999; Tinoco and Vazquez-Duhalt, 1997; Wang *et al.*, 1999). Addition of the amphiphilic polymer presumably prevents dehydration of the enzyme as the hydrophilic portion of these groups is strongly hydrated. The hydrophobic portion of amphiphilic polymers like PEG may also increase the solubilities of enzymes in organic solvents. Recently, Jene *et al.* (1997) have used the polymer polyoxyethylene(23)lauryl ether (Brij35), activated with cyanuric chloride, to increase the stability and activity of a catalase in organic solvents. Typically, the binding of these groups does not significantly alter the structure of the protein (Khmelnitsky *et al.*, 1988; Koops *et al.*, 1999) although Tinoco and Vazquez-Duhalt (1997) noted an increase in the substrate range of cytochrome c after modification with PEG, indicating the possibility of a structural change. An advantage of using this system to effect an increase in stability in organic solvents is the fact that enzymes retain their solubility; this allows for straightforward kinetic investigations. However, this solubility makes recovery during industrial processes difficult. A possible solution may be found in the work of Ito *et al.* (1999), who have modified subtilisin with a photoresponsive copolymer whose solubility can be regulated by irradiation with light. Irradiation with UV light renders the enzyme insoluble and it can be easily recovered by centrifugation; subsequent irradiation with visible light converts the enzyme back to a soluble form.

1.7 Objectives

The overall objectives of this study were to identify strains of fungi capable of producing large amounts of laccase and use them to make milligram quantities of the

enzyme. These enzymes would then be characterized and modified for better activity in organic solvents, with a final goal of improving rates of PAH oxidation.

2 Methods and Materials

2.1 Fungal Strains

Many fungal strains were examined in the early stages of the screening work, collected from several sources. *Pleurotus ostreatus* ATCC 58053, ATCC 44309 and ATCC 34676 were obtained from the American Type Culture Collection, Manassas, VA, USA. *Myxotrichium arcticum*, *Pseudogymnoascus roseus*, *Pseudogymnoascus frigida* and *Ovadendron sulfureo-ochracum* were generous gifts from Trevor Lumley during his mycological research at the University of Alberta. *Pleurotus ostreatus* UAMH 7988, UAMH 7961, UAMH 7976, UAMH 7964, UAMH 7972, UAMH 7980, UAMH 7992 and *Coriolopsis gallica* UAMH 8260 were obtained from the University of Alberta Microfungus Collection and Herbarium, Devonian Botanical Gardens, Edmonton, AB, Canada. Several dry spore suspensions and fruiting bodies of white-rot fungi were collected from Whitemud Park, Edmonton, AB, Canada. The strains were initially grown on potato dextrose agar (PDA; Difco, Detroit, MI) at 28 °C for 7 days. After initial growth on PDA, mycelia were excised and transferred to 3% Kellog's Bran Flakes (KBF) (Kellogg Canada Inc., London, ON) 1.5% agar plates and incubated at 28 °C. After growth for one week, the stock plates were stored at 4 °C and transferred to fresh plates every four months.

2.2 Agar-Based Screening

2.2.1 Tannic Acid

All of the collected strains were grown on agar medium containing tannic acid as the sole carbon and energy source. Tannic acid (5 g) (MCB Manufacturing Chemists Inc., Cincinnati,

OH) was dissolved in 200 mL distilled water; 5 g malt extract (Difco) and 20 g agar (Difco) were dissolved in 800 mL water. Both solutions were autoclaved, cooled to ca. 45 °C, then mixed together vigorously and used to pour petri plates containing about 20 mL of medium. A 1 cm^2 area of fungal mycelia was used to inoculate the center of each plate and the plates were incubated at 28 °C. Oxidation of the tannic acid in the medium was followed by observing the resultant darkening of the medium adjacent to the inoculation site and subsequent growth.

2.2.2 Remazol Brilliant Blue R

The dye Remazol Brilliant Blue R (RBBR) (Sigma, St. Louis, MS) was incorporated into agar medium as follows: 10 g ground KBF, 7.5 g agar (Difco) and 0.15 g RBBR were mixed well into 500 mL of distilled water and autoclaved. After autoclaving, the solution was vigorously stirred to resuspend the ground bran flakes and then poured to petri plates before resettling or cooling. The resultant agar had a deep blue colour. Zones of colour change to white around the fungal hyphae inoculum of 1 cm^2 (and resulting growth) were compared.

2.3 Screening of Selected Cultures in Liquid Media

Selected strains were tested for their ability to produce extracellular oxidative enzymes in a variety of media. Media compositions were as follows:

Glucose-Malt Extract-Yeast Extract (GMY) (per L): 10 g glucose (BDH), 3.5 g malt extract (Difco), 2.5 g yeast extract (Difco), 2.0 g KH_2PO_4 (BDH), 0.5 g $MgSO_4 \bullet 7\ H_2O$ (BDH).

Kellog's Bran Flakes (KBF): 30 g Kellog's Bran Flakes were ground in a Waring blender to a fine consistency and suspended in 1 L 0.6 M potassium phosphate buffer pH 6.0 for a 3%

w/w final solution. Similar methods were used to prepare Wheat Bran (WB), Oat Bran (OB) and Rice Bran (RB) media.

2.3.1 Inoculum Preparation

An area of fungal mycelia (1 cm^2) was excised from KBF maintenance plates and homogenized with GMY medium using a Sorvall Omni-Mixer at speed 7 for 15 s or until completely homogenized. This homogenized medium (200 mL) was incubated at 28 °C in a 500 mL Erlenmeyer flask at 200 rpm for 5 d or until significant pelleted growth had occurred. The inoculum culture was then again homogenized and used to inoculate different media at 5% v/v. All shake flask production experiments were performed in triplicate.

2.3.2 Induction Experiments in Liquid Media

Incubations were constructed as described above. After 48 h of incubation, filter-sterilized solutions of the inducers 3,4-dimethoxybenzyl alcohol (veratryl alcohol), 2,6-dimethylbenzenamine (xylidine), and 4-hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde) were added to predetermined concentrations (all obtained from Sigma).

2.3.3 Static Incubation

Five hundred milliliters of medium was inoculated at 5% v/v in a 2 L Erlenmeyer flask with 200 mL of RB medium and incubated without shaking at 28 °C; triplicate flaskes were inoculated.

2.3.4 pH of Media

The pH of media were adjusted by utilizing phosphate buffer at the appropriate pH. Phosphate buffer in growth experiments was used at 0.06 M in all cases; this concentration

was sufficient to maintain pH in uninoculated controls throughout autoclaving and the duration of all experiments.

2.4 Enzyme Production

2.4.1 2L Scale-Up

Media preparation, inoculation and incubation were as above but 2 L Erlenmeyer flasks containing 500 mL of medium were used.

2.4.2 Stirred Tank Reactor Scale-Up

Different media compositions, volumes, inducers, impeller types and placements were used to compare enzyme production in a New Brunswick Scientific Microferm fermentation unit (14 L capacity, 10 L working volume). The inoculum train for both *P. ostreatus* ATCC 58053 and UAMH 7988 was as follows: 1 cm^2 of hyphae from a KBF maintenance plate was excised and homogenized with 100 mL of GMY which was then incubated for 5 days at 200 rpm and 28 °C. After 5 days, the contents of the flask were homogenized again and used to inoculate 500 mL of GMY in a 2 L flask. This flask was then again incubated for 5 days at 200 rpm and 28 °C. The entire contents of the 2 L flask were used to inoculate the fermenter.

2.5 Purification

Harvested cultures were filtered through finely woven cotton fabric to remove the bulk of biomass and solid bran media and were immediately frozen at –20 °C. Once thawed, the culture filtrate was centrifuged (10 000 x g, 15 min) and paper filtered to remove any insoluble material. The supernatant was concentrated using a Millipore cellulose cartridge (10 kDa cutoff) to ca. 10% of the initial volume. Polyethyleneimine (PEI) was stirred in to a final concentration of 0.15%; the resultant solution was then centrifuged rapidly (20 000 x g, 20 min) to pellet the precipitated PEI and bound material. The clarified supernatant was then

concentrated further (to ca. 5% of the original volume) and extensively dialyzed using an Amicon ultrafiltration apparatus with a 30 kDa cutoff (YM30) filter.

This concentrate was then applied to a DE-52 column (2.5 cm x 50 cm) and eluted with a linear gradient from 0 to 0.5 M NaCl in 10 mM phosphate buffer (pH 6). Fractions containing the dominant isoform were pooled, dialyzed, and concentrated by ultrafiltration using a 30 kDa cutoff membrane (YM30).

The laccase preparation was further purified utilizing a Sephadex G 100 column; the highly concentrated sample was applied and eluted using 0.1 M NaCl in 10 mM phosphate buffer (pH 6). The fractions containing significant amounts of laccase relatively free of chromophore contamination were pooled, concentrated and dialyzed as above.

Final purification was achieved using FPLC with a Mono Q column. The concentrated sample was applied and eluted with the following scheme where solvent a= 10 mM phosphate buffer (pH 6) and solvent b= 1 M KCl in 10 mM phosphate buffer (pH 6): 0-5 min: 100% a, 5-60 min: 10% b to 30% b, 60-75 min: 30% b to 100% b, 75-80 min: 100% b, 80-90 min: 100% b to 0% b. Where solvent b is used as a fraction solvent a made up the remainder of the solution. Fractions containing laccase activity free from contamination were pooled, dialyzed and concentrated using a Millipore ultrafree-4 centrifugal filter unit, with a 10 kDa cutoff.

2.6 Characterization

2.6.1 pH Optimum

Concentrated (10 mM) 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) solution in water was added to 890 µL of the appropriate buffer for the chosen pH. Purified enzyme (10 µL) was added to and the initial, linear reaction rate was followed. Buffers were

chosen as follows: pH 2-2.5: citric acid – sodium citrate; pH 3-5.5: sodium acetate – acetic acid; pH 6-8: potassium phosphate; pH 9: Tris – HCl.

2.6.2 pH Stability

An enzyme dilution (25 µL) in water was mixed with 225 µL of buffer and incubated at room temperture (22 °C). An aliquot of the suspension (20 µL) was removed periodically and assayed using 980 µL of 1 mM ABTS in 0.1 M sodium acetate buffer (pH 4) and compared with controls utilizing the same amount of enzyme without incubation. Buffers utilized were the same as those in section 2.6.1.

2.6.3 Temperature Optimum

ABTS (1 mM) in 0.1 M sodium acetate buffer (pH 4) was preheated and held at the desired temperature using an internal cuvette holder set at a controlled temperature by an external water bath. Once at temperature, a constant amount of enzyme was injected and the temperature of the assay solution was held constant during the brief measurement by the cuvette holder (15 sec integration). All temperatures tested provided linear results over the initial 15 sec after injection of enzyme.

2.6.4 Temperature Stability

The method employed was the same as in section 2.6.2 but 0.1 M sodium acetate buffer (pH 4) only was utilized at incubation temperatures of 4 °C, 22 °C and 60 °C.

2.6.5 Gel Electrophoresis

2.6.5.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as follows: 4% stacking and 15% separating gels were used; voltage was set at 100 V for the first 10 min and 180 V thereafter. Staining was performed with either a Bio-Rad Coomassie or Silver Stain Kit. Molecular weight standards were purchased from Sigma as a

group. Loading buffer included 50% β-mercaptoethanol and 0.01% bromophenol blue in glycerol.

2.6.5.2 Non-denaturing gel electrophoresis was performed under the same conditions as the preceding section, except for the following: samples were not boiled and were loaded using 50% glycerol only as loading buffer. Activity staining was accomplished by incubation with o-dianisidine (1 mM) in 50% methanol.

2.6.5.3 Isoelectric focussing was performed with pre-cast gels (pH 3-10) purchased from Bio-Rad. Anode buffer (7 mM phosphoric acid) and cathode buffer (20 mM lysine, 20 mM arginine) were also purchase from Bio-Rad. Samples were loaded with 50% glycerol. Constant voltage was applied as follows: 100 V, 7 mA, 1 hour; 250 V, 7 mA, 1 h; 500 V, 6 mA, 30 minutes. Protein bands were visualized with Bio-Rad IEF Stain (Coomassie R-250 and Crocein Scarlet) or silver staining. IEF standard proteins were purchased from Sigma or previously purified in this laboratory.

2.7 Enzyme Assays

2.7.1 Laccase activity was routinely determined by following the oxidation of ABTS (Wolfenden and Wilson, 1982). The reaction mixture contained 1 mM ABTS in 0.1 M sodium acetate buffer, pH 4 or 5 to 50 µL of enzyme sample. The reaction was followed at 30 °C and at 436 nm (ε_{436} = 29 300 M^{-1} cm^{-1}).

2.7.2 Lignin peroxidase activity was measured by following the H_2O_2 dependent oxidation of veratryl alcohol to veratraldehyde at 30° C. Reaction mixtures contained 4 mM veratryl alcohol in 40 mM succinate buffer, pH 3, and were initiated by the addition of H_2O_2 to a final concentration of 0.4 mM. Absorbance was monitored at 310 nm (ε_{310} = 9 300 M^{-1} cm^{-1}).

2.7.3 Manganese peroxidase activity was measured by the H_2O_2-dependent formation of oxidized manganic-malonate complex at 270 nm (ε_{270} = 11 590 M^{-1} cm^{-1}). Reactions contained 1 mM manganous sulfate in 50 mM malonate buffer (pH 4.5) and 5 to 50 µL of enzyme sample and were initiated by the addition of H_2O_2 to a final concentration of 0.1 mM (Wariishi *et al.*, 1989). One enzyme unit was calculated as 1µm of substrate oxidized per min.

2.8 Substrate Specificity

Portions of 995 µL of 5 mM solutions of p-aminophenol, tolidine, veratryl alcohol, catechol, guiacol, hydroquinone and 2,6-dimethoxyphenol in 0.1 M sodium acetate buffer (pH 6) were injected with 5 µL of concentrated enzyme and the reaction was followed spectrophotometrically from 200 to 800 nm. Syringaldazine was also utilized but solubility demands dictated the additional presence of 10% ethanol; parallel study with ABTS as the substrate indicated that this concentration of ethanol had no discernable effect on the reaction.

2,6-Dimethoxyphenol, ABTS, 4-aminophenol, catechol and guaicol were chosen for further kinetic study. Stock solutions of each substrate were prepared at 50 mM in 0.1 mM pH 4 sodium acetate buffer and diluted with acetate buffer to the final concentrations needed to determine K_m and V_{max}. At least six data points were collected over the range of the Michaelis-Menten curve; K_m and V_{max} were calculated using nonlinear regression (see Appendix). Measurements at each concentration were taken in duplicate.

2.9 Effects of Solvent on Catalysis

2.9.1 Enzyme Stability

Enzyme solution (30 µL) was incubated with 0.2 M sodium acetate buffer (pH 4) or 0.2 M Tris-HCl buffer (pH 9) and the desired amount of organic solvent to make a final volume of 500 µL. Samples (80 µL) were periodically taken and assayed for activity in 5 mM ABTS in 0.1 M sodium acetate buffer (pH 4). The results were compared against the activity of 4.8 µL of unincubated enzyme in the same assay solution.

2.9.2 Enzyme Kinetics

Concentrated stock solutions of ABTS (up to 80 mM) were prepared and portions diluted with organic solvent and buffer to the desired final ABTS and solvent concentrations in 995 µL. Enzyme solution (5 µL) was added and the initial, linear reaction rate was followed.

2.10 PAH Oxidation

Solutions (20 µM) of each PAH examined were prepared using 15% acetonitrile. Ten microlitres of enzyme solution (ca. 0.5 units in each case) was added to 990 µL of the PAH solution and incubation took place for 10, 20 and 30 min. At these time points, acetonitrile was added to a final concentration of 60% to stop enzyme activity. Samples (15 µL) were then analyzed by HPLC and compared to controls with boiled enzyme. These assays were performed in triplicate.

2.11 HPLC Separation

A Waters-Millipore system including a WISP 712 automatic injector and an analytical reverse phase column (Spherisorb 10, RP-18 10 µm, 100 x 4.6 mm) were used and attached to a model 486 Tunable Absorbance detector. Information from the absorbance detector was recorded and peak areas calculated by a Hewlett-Packard 3392A integrator. Each separation and quantification of PAH oxidation products was preceded by a standard curve

of the PAH. Compounds were eluted isocratically with a mobile phase of 60:40 acetonitrile:water.

2.12 Chemical Modification of Enzymes

2.12.1 Brij35

Brij35 (Sigma) modification was based on the method of Jene *et al.* (1997). Activated Brij35 was synthesized as follows: 0.38 g of cyanuric chloride was added to 200 mL of dry toluene that contained 13 g of solid anhydrous sodium carbonate, then 4.7 g of Brij35 was added immediately. The mixture was heated to 75 °C and stirred for 125 h at this temperature. A reflux apparatus prevented evaporative loss of the toluene. After 125 h, the solution was removed from heat and centrifuged (3000 rpm for 10 min) to remove insoluble solids. The remaining product was rapidly concentrated to ca. 10 mL by rotary evaporation at 45 °C. The activated Brij35 product was precipitated on the addition of 100 mL of 4 °C petroleum ether. The remaining solvent mixture was removed under vacuum and the gelatinous activated Brij35 was stored at –20 °C.

Modification of laccase with activated Brij35 was performed as follows. A solution of laccase (1 mg) in 5 mL 0.4 M potassium borate buffer (pH 9.0) at 22 °C was placed in a polypropylene tube containing 33 mg of activated Brij35. The mixture was tumbled at 22 °C for 120 min. The enzyme solution was then diluted with 50 volumes of water and then extensively dialyzed via ultrafiltration with a YM30 membrane. The filtrate was monitored for excess, unreacted activated Brij35 using the method of Milwidsky and Gabriel (1982). A 1 mL sample of the filtrate was added to 1 mL of cobaltothiocyanate reagent and 1 mL of dichloromethane. The mixture was vortexed for 1 min and then centrifuged (10 000 x g for 1 min) to separate the two phases. The absorbance of the dichloromethane layer was read at

640 nm and compared against controls. When no more free, unreacted activated Brij35 was found in the filtrate, dialysis was stopped and the sample concentrated to ca. 10 mL.

2.12.2 PEG

Laccase modification with PEG followed the method of Vazquez-Duhalt *et al.* (1992). Methoxypolyethylene glycol activated with cyanuric chloride was purchased from Sigma. One milligram of laccase and 33 mg of activated PEG were mixed with 5 mL of 0.4 M potassium borate buffer (pH 9). The mixture was tumbled at 22 °C for 120 min. In a manner identical to the preceding section, excess unreacted activated PEG was removed via ultrafiltration.

2.13 Analytical Methods

2.13.1 Protein Content

Protein content was determined using bovine gamma globulin as the standard by the method of Bradford (1984). The dye-binding reagent was purchased from BioRad and samples were compared against concurrently prepared serial dilutions of the standard.

2.13.2 Reducing Sugar Content

Reducing sugar content was measured using dinitrosalicylic acid (DNS) with D-glucose as the standard. DNS reagent (1 mL) was mixed well with 200 µL of the sample to be tested and 800 µL of 0.1 M sodium citrate buffer (pH 4.8). The mixture was heated to 90 °C for 10 min and then cooled to 22 °C. Five millilitres of water were mixed in and the absorbance of the resultant solution at 550 nm was determined.

2.13.3 Total Carbohydrate

Total carbohydrate was measured using the method of Dubois *et al.* (1956) with D-glucose as the standard.

2.13.4 Amino Groups

Free amino group determination was made using trinitrobenzenesulfonic acid with the method of Habeeb (1966).

2.13.5 Copper Content

Copper content was measured by the method of Felsenfeld (1960) using 2,2'-biquinolone as the titrating reagent.

2.13.6 Mass Spectrometry

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis for molecular weight was performed as follows. The matrix used in these experiments was α-cyano-4-hydroxycinnamic acid (HCCA). HCCA purchased from Aldrich was first purified by recrystallization from ethanol. A two-layer MALDI deposition method was used. This involves the deposition on the probe tip of a microcrystalline matrix layer via fast evaporation from a 0.7 μL solution of HCCA (12 mg/mL) dissolved in 80% acetone/methanol. One microlitre of the sample was mixed with either 1 or 9 μL of a saturated HCCA solution in 40% methanol/0.1% TFA in water. The solution was briefly vortexed and a 0.4 μL portion was deposited on top of the first matrix layer. Once dried, the spot was washed three times with 0.75 μL of room temperature water.

The samples were analyzed with a Hewlett Packard LD-TOF system, equipped with delayed extraction. Data processing was done with the IGOR Pro software package (Wavemetrics Inc., Lake Oswego, OR).

2.13.7 N-Terminal Sequencing

N terminal sequencing and total amino acid content determinations were performed by the Alberta Peptide Institute, University of Alberta, Edmonton, AB.

3 Results

The work performed in the completion of this research can be divided into three parts, each with distinct goals. The first subsection (3.1) deals with the production of laccase and is primarily concerned with strain selection and optimization of growth conditions. The second major part of these results (3.2) illustrates the purification and characterization of the laccases produced with the strains and methods chosen in subsection 3.1. The final subsection (3.3) describes the modification of these enzymes and the effect of these procedures on their activity in organic solvents, with a resultant increase in utility for the oxidation of PAHs.

3.1 Strain Screening and Production Conditions

The initial experiments were designed to isolate fungi capable of producing significant amounts of laccase, as an important first step in producing the large amounts of this enzyme required for further study. These experiments involved the agar-based screening of a number of isolates and liquid growth studies of a subset.

3.1.1 Agar-Based Screening

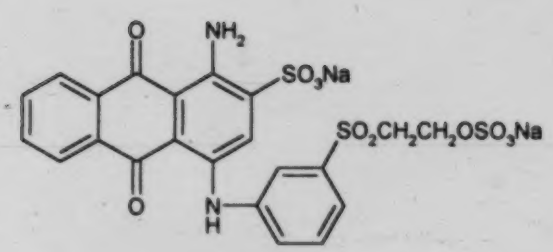
Two types of agar-based screens were utilized for the determination of laccase production. Both of these techniques utilized compounds with aromatic character that typically change colour with oxidation. Production of laccase and its subsequent extracellular secretion to the surrounding medium could be expected to produce such an easily scored and quantifiable colour change. The two compounds utilized in these experiments, Remazol Brilliant Blue R (RBBR) and tannic acid, are illustrated in Figure 7. Inoculation of the plates

with constant amounts of hyphae excised from KBF maintenance plates gave repeatable and easily demonstrable results. Growth of several strains produced a characteristic darkening of the medium after inoculation on tannic acid agar; however, some strains failed to produce any change in the medium. An example of the colour change generated can be seen in Figure 8 (A). Conversely, growth on RBBR agar resulted in a clearing of colour from the medium, leaving only the residual hue of the agar base. An example of this clearing effect can also be seen in Figure 8 (A). In Figure 8 (B), it is possible to see the highly ramified network of hyphae extending from the initial site of inoculation that was typical of many of the strains inoculated onto RBBR agar; this effect was much less pronounced during growth on tannic acid agar.

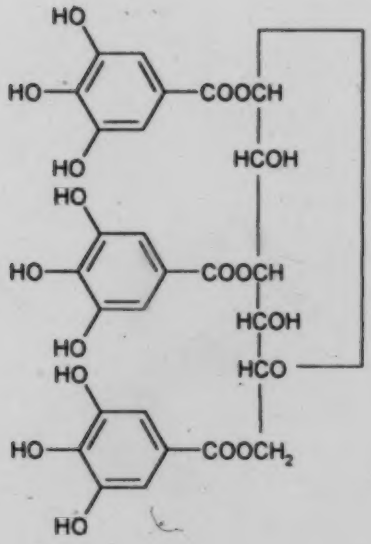
The quantified results of these agar-based screenings can be seen in Table 6. Strains that produced strong colour changes in both media were *Coriolopsis gallica* UAMH 8260 and *Pleurotus ostreatus* ATCC 58053 and UAMH 7988, while a number of other *P. ostreatus* strains also produced colour changes to varying degrees in the media. The first six strains in the table were isolated from charred wood by T. Lumley; their failure to oxidize the compounds is somewhat surprising. Similarly, the individually collected woods-isolates also failed to produce any significant change in medium colour.

3.1.2 Screening in Liquid Media

Based on the results described in § 3.1.1, study was focussed on several strains of *P. ostreatus* and further work was performed looking at laccase production in liquid media. Earlier work by Pickard *et al.* (1999) demonstrated the utility of cereal bran based media for the production of laccase, so KBF medium was utilized in these initial experiments.

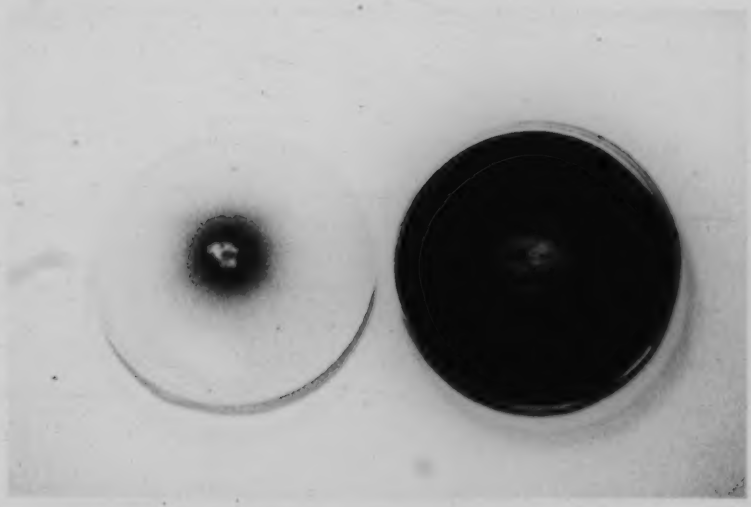


Remazol Brilliant Blue R



Tannic acid (corilagin)

Figure 7: Structures of Remazol Brilliant Blue R and tannic acid



(A)

(B)

Figure 8: (A) Tannic acid agar plate (left) and RBBR agar plate (right) three days after inoculation with a strain of *Pleurotus ostreatus.*
(B) Oblique angle view of RBBR agar plate seen in (A)

Table 6: Agar-based screening results

[illegible]	Lanna Azul	Remazol Brilliant Blue R
Ovadendron sulfurco-ochraceum	-	-
Pseudogymnoascus roseus F681-03-SIE 3-7 A	-	-
P. roseus ML 07 SIJ 11-17 E	-	-
Pseudogymnoascus frigida F953-03-S2C 7-16A	+	+
P. frigida F953-03-S2C	+	+
Myxotrichum arcticum	-	-
Woods isolate	-	-
Coriolopsis gallica UAMH 8260	++	++
Pleurotus ostreatus UAMH 7961	+	-
P. ostreatus UAMH 7964	++	+
P. ostreatus UAMH 7972	+	++
P. ostreatus UAMH 7976	+	++
P. ostreatus UAMH 7980	++	+
P. ostreatus UAMH 7988	++	++
P. ostreatus UAMH 7992	+	++
P. ostreatus UAMH 7363	-	+
P. ostreatus ATCC 58053	++	++
P. ostreatus ATCC 44309	+	+
P. ostreatus ATCC 34676	+	+

Key: - = no colour change in medium
+ = 0.1 to 2 cm diameter zone of colour change after 3 days
++ = greater than 2 cm diameter zone of colour change after 3 days

Table 7 demonstrates the maximum titer of laccase production from 12 *P. ostreatus* strains in KBF medium alone (maximum uninduced titer). The production values seen in the second column of Table 7 separate the fungi tested into three categories. 1) Production by strain ATCC 58053 clearly puts this fungus in a category by itself, with approximately 6 times the production of strain UAMH 7961, the second highest producer. 2) Strains UAMH 7961, UAMH 7988, ATCC 44309, and ATCC 34676 comprise a second category of moderate producers with a typical maximum uninduced titer of around 2 EU/mL. 3) The remaining strains in the table were poor producers of laccase despite similar growth characteristics to the higher producing strains. In agitated culture, all tested strains demonstrated a pelleted growth morphology that appeared to be required for enzyme production. Several strains were also examined for the ability to produce laccase in liquid culture but under static incubation conditions. In each case, the level of production was at least ten-fold lower than that achieved in agitated cultures (data not shown).

3.1.2.1 Induction of Laccase Production during Liquid Screening

Several researchers have reported significant increases in laccase production in liquid media upon the addition of small molecular weight inducing compounds. Veratryl alcohol, xylidine and syringaldehyde were tested for their ability to induce laccase production in an effort to increase overall yields. The effect of these compounds is often concentration dependent; therefore, a variety of concentrations were tried in a range finding experiment to determine the optimum concentration for induction. These compounds were initially utilized with *P. ostreatus* UAMH 7988 at 2, 4, 5, 6 and 8 mM concentrations, added after two days of incubation (because the toxicity of these compounds can inhibit growth). While syringealdehyde was incapable of induction above baseline levels (data not shown), xylidine

Table 7: Maximum laccase production by *P. ostreatus* strains (induced and uninduced values)

P. ostreatus Strain	Max. uninduced titre (I.U. mL^{-1}) [± SD][1]	Max. induced titre (I.U. mL^{-1}) [± SD][2]
ATCC 58053	12.2 ± 0.8	15.6 ± 1.6
UAMH 7961	2.1 ± 0.1	4.3 ± 0.3
UAMH 7988	1.5 ± 0.2	4.2 ± 0.7
ATCC 44309	2.0 ± 0.2	3.4 ± 0.3
ATCC 34676	2.7 ± 0.05	3.2 ± 0.3
UAMH 7989	0.2 ± 0.03	1.8 ± 0.2
UAMH 7363	0.8 ± 0.04	0.9 ± 0.04
UAMH 7964	0.1 ± 0.03	0.6 ± 0.05
UAMH 7972	0.4 ± 0.18	0.3 ± 0.08
UAMH 7976	0.09 ± 0.02	0.2 ± 0.03
UAMH 7992	0.05 ± 0.00	0.06 ± 0.02
UAMH 7980	0.02 ± 0.00	0.03 ± 0.00

[1]Maximum uninduced values obtained in 3% KBF medium
[2]Maximum induced titers obtained in 3% KBF medium after induction with 2 mM xylidine on day 2 of incubation

and veratryl alcohol were successful in a concentration dependent manner. These results are seen in Figure 9 and Figure 10 respectively. The induction effect of xylidine appeared more potent, producing a titer of 4.2 EU/mL at 2 mM compared to 2.8 EU/mL for veratryl alcohol at 2 mM.

Further, a broader range of xylidine concentrations was used to test the potential of significantly higher and lower concentrations for induction capability. The results of this study are seen in Figure 11; again, the best results are seen in the 1 mM range, with laccase production dropping off sharply with the tested tenfold increases and decreases.

Table 7 also demonstrates the maximum induced titer of laccase in liquid culture using an amendment of 2 mM xylidine. Laccase production increased significantly with this treatment with the first six strains tested in Table 7; however, the addition of xylidine did not increase production in the lower producing strains occupying the bottom six positions of Table 7.

As a result of the above experiments, two strains were chosen for further study. An obvious choice was *P. ostreatus* ATCC 58053 for its prodigious laccase production; *P. ostreatus* UAMH 7988 was also chosen as a source of another laccase primarily for comparison and contrast purposes in later studies. Figure 12 illustrates the morphology of these two fungi grown on potato dextrose agar (PDA) for seven days.

3.1.3 Screen of Bran Sources for Production Media

Since the type of media used can also play an important role in determining enzyme production, different sources of bran were also tested for the ability to support laccase production. *P. ostreatus* ATCC 58053 was incubated in buffer solutions (pH 6) containing 3% Kellog's Bran Flakes (KBF), rice bran, wheat bran or oat bran. The resultant laccase titers

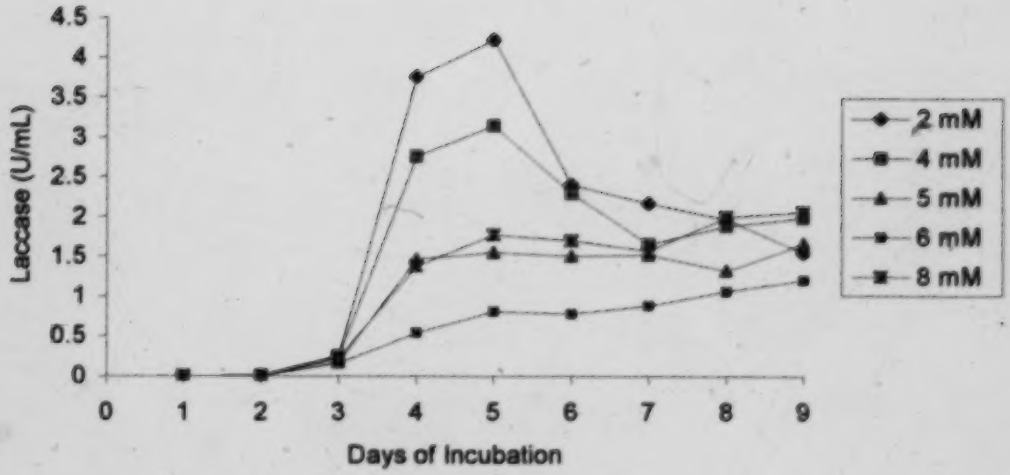


Figure 9: Xylidine based induction of laccase production in *P. ostreatus* UAMH 7988. Numbers in the legend refer to the concentration of xylidine used to induce production, added on day 2 of incubation

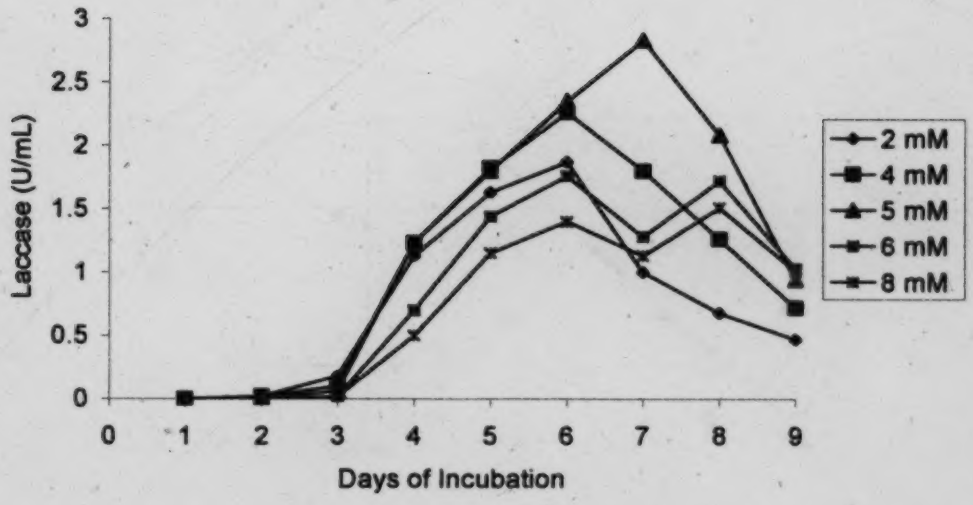


Figure 10: Veratryl alcohol based induction of laccase production in *P. ostreatus* UAMH 7988. Numbers in the legend refer to the concentration of veratryl alcohol used to induce production, added on day 2 of incubation

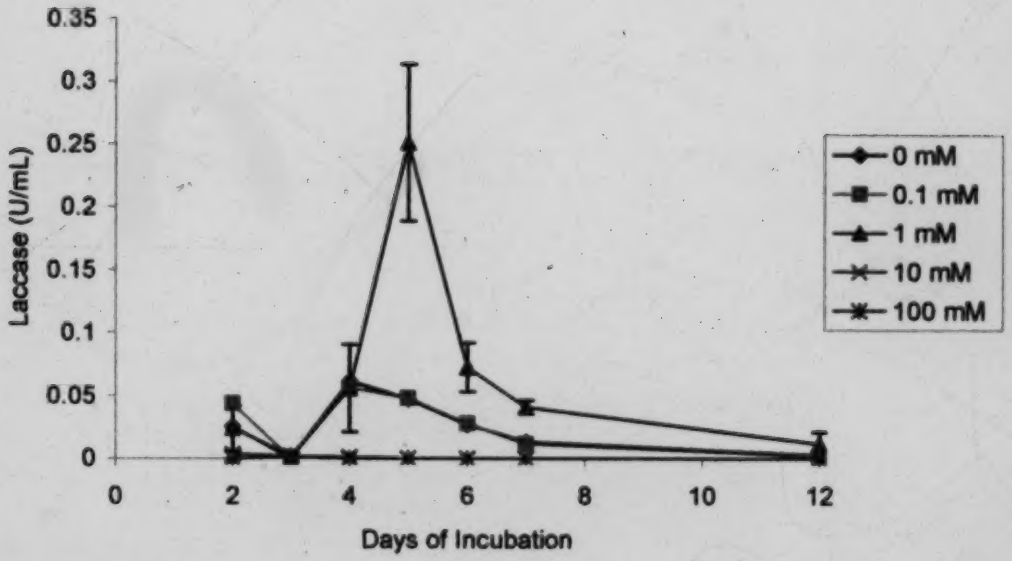
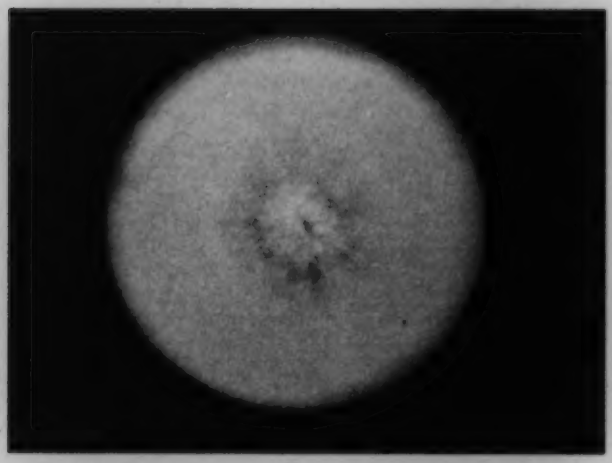


Figure 11: Broad range xylidine induction of laccase production in *P. ostreatus* UAMH 7988. Error bars at 1 mM extend ± 1 SD.



(A)

(B)

Figure 12: (A) *P. ostreatus* ATCC 58053 grown on PDA: after 7 days there is confluent growth to the margin of the petri dish.

(B) *P. ostreatus* UAMH 7988 grown on PDA: after 7 days growth has reached the edge of the plate but hyphal cover is less thick than ATCC 58053.

can be seen in Figure 13. The maximum production is seen with rice bran, although the production in KBF is not significantly different. Production was lower in oat bran even though this media supported growth in a similar manner to KBF and rice bran. Wheat was an extremely poor source of bran to support either growth or laccase production.

3.1.4 Initial pH of Medium and Laccase Production

Medium pH was also assessed as a factor influencing production. Phosphate buffers at different pHs were examined as suspension milieu for 3% KBF media. The results on laccase production with *P. ostreatus* ATCC 58053 can be seen in Figure 14. Maximum production is seen with pH 6.5, however, the values for pHs 5 to 6.5 are very similar. Production with media at pH 7 also reached similar values, but only after a one-day lag.

3.1.5 pH of Medium During Incubation

In the experiment detailed in § 3.1.4, the daily pH values of the individual flasks were also monitored. As can be seen in Figure 15, the pH of each media drifted towards 6.0 over the duration of the experiment, regardless of the initial pH tested. KBF media in each of the buffer systems alone (without inoculation with fungi) did not exhibit this trend.

3.1.6 Temperature of Incubation

The temperature of incubation of *P. ostreatus* ATCC 58053 was also altered and the effects on laccase production noted. Figure 16 illustrates the results of this experiment. The highest production was seen at 28 °C, with notable production at 21 °C as well. Higher temperatures than these (30 °C and 37 °C) supported poor fungal growth and laccase production.

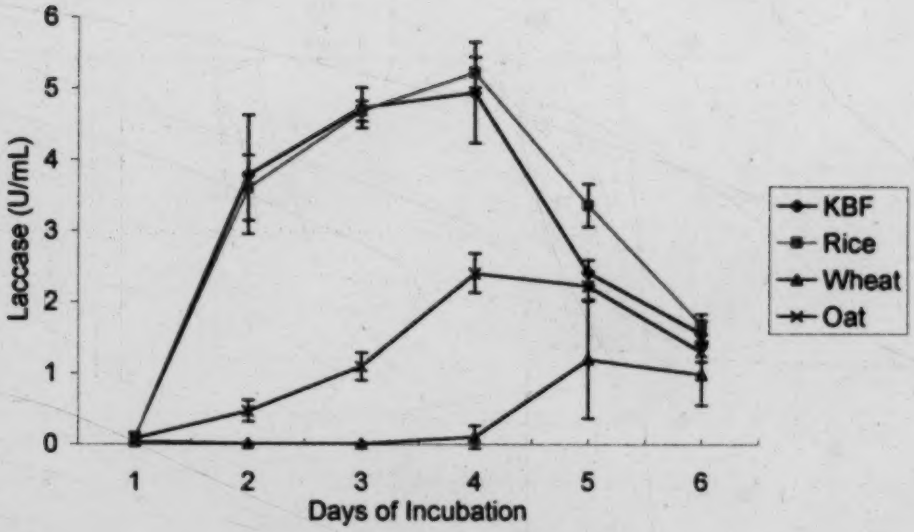


Figure 13: Screen of different bran sources for laccase production with *P. ostreatus* ATCC 58053

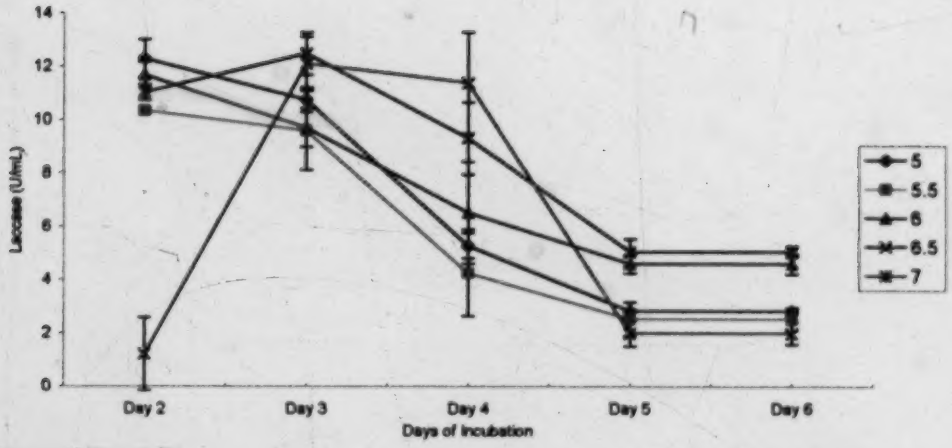


Figure 14: Effect of medium pH on laccase production by *P. ostreatus* ATCC 58053

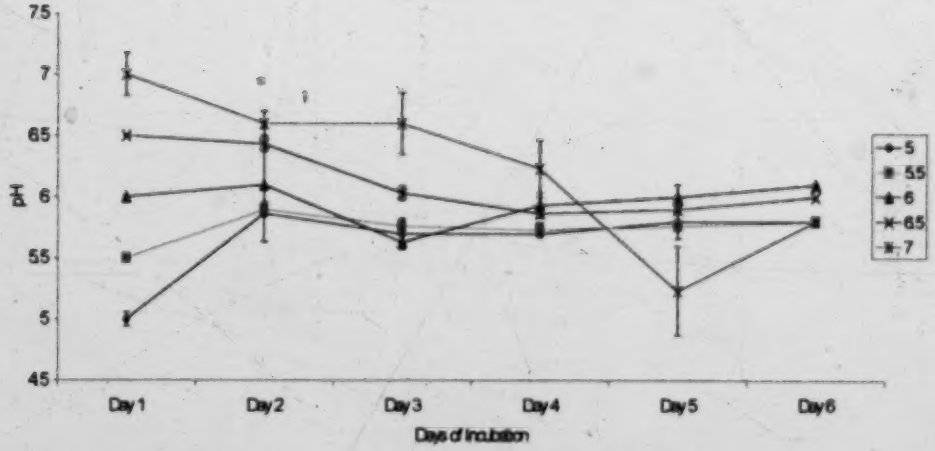


Figure 15: Medium pH changes during growth and laccase production by *P. ostreatus* ATCC 58053 with different initial buffering points

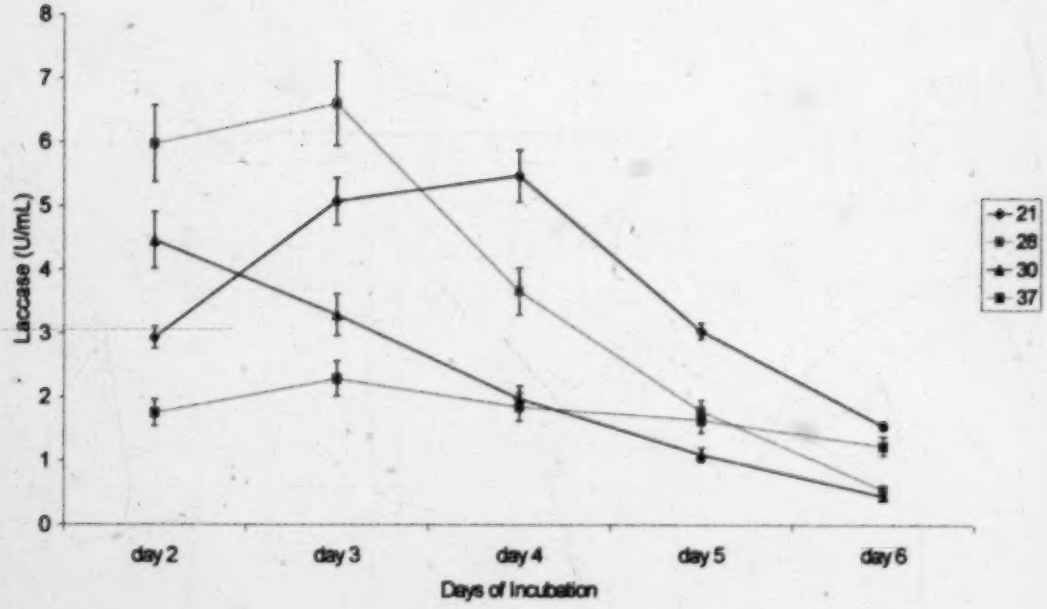


Figure 16: Effect of incubation temperature on laccase production with *P. ostreatus* ATCC 58053. Numerals in the legend refer to degrees Celsius.

3.1.7 2 L Scale-Up

The optimal production conditions determined in § 3.1.3 to § 3.1.6 (rice bran medium, initial pH 6.5, temperature 28 °C) were utilized in a scale-up to production in 2 L Erlenmeyer flasks (as opposed to the earlier 500 mL flasks) with and without induction with xylidine for *P. ostreatus* ATCC 58053 and UAMH 7988. Values of laccase production were similar to those seen in the 500 mL shake flask experiments. ATCC 58053 produced 13.6 ± 2.2 U/mL in uninduced culture and 16.9 ± 1.2 U/mL in xylidine induced (2mM) culture; UAMH 7988 produced 2.7 ± 1.4 U/mL in uninduced culture and 3.7 ± 0.8 U/mL in xylidine induced (2mM) culture. While production in 2 L or other flasks is feasible for small-scale studies, the only way to produce enough homogenous enzyme for the following work was in a larger scale fermentation system.

3.1.8 Fermentation

Several attempts were made at designing an optimal fermentation protocol for the production of laccase from the chosen *P. ostreatus* strains. Each of the initial trials was performed with *P. ostreatus* ATCC 58053 because of its capability to produce large amounts of laccase: the success of a run could be more easily judged.

Initially, a stirred tank fermenter was used without an impeller (to alleviate anticipated shear stress) and an elevated air flow to convert it to an airlift type of fermenter; this trial was run with 3% KBF and induced with 2 mM xylidine on day 2 of the incubation. Production in this fermeter peaked at only 0.8 U/mL. Much of the heavy bran material and

fungal matter could not be circulated in the larger working volume (10 L in a 14 L chamber) and settled at the bottom, presenting obvious mass transfer limitations.

Secondly, the same system was utilized but with the addition of a marine impeller at 200 rpm and a lower airflow rate. Production in this system only reached 1.3 U/mL; further, overall pelleted growth was poorer than observed in either of the shake flask systems. Although the scaled volumes were identical, the addition of xylidine also appeared to have a negative efffect on growth; further, the fact that xylidine is highly toxic argues against its use in these systems.

Thirdly, another stirred tank fermenter was set up utilizing 3% KBF medium and a marine impeller at 200 rpm, without any xylidine spike. Production in this fermenter rose to 3.8 U/mL and pelleted growth appeared much earlier and with more vigor than in the earlier trials. However, at 10 L of volume, significant foaming in the vessel led to "foam outs" where some of the fermentation material escaped from the vessel; in addition to losing production, such events can also lead to contamination. The addition of commercial antifoam compounds, like PEG 5000, could possibly complicate later purification of the enzyme, so this option was discarded. Instead, another trial was attempted with a lower volume of material in the fermentation vessel.

The fourth trial utilized 8 L of 3% rice bran in the 14 L fermenter, with a single marine impeller at 200 rpm and an airflow of 2 L/min. Production in this system exceeded that of the uninduced culture in shake flasks at 14.7 U/mL. A strong but transient peak of laccase production was observed on day 3 of the incubation, similar to shake flask experiments. A photograph of an inoculated and operating fermenter system can be seen in Figure 17. Note the pelleted growth being circulated by the impeller.

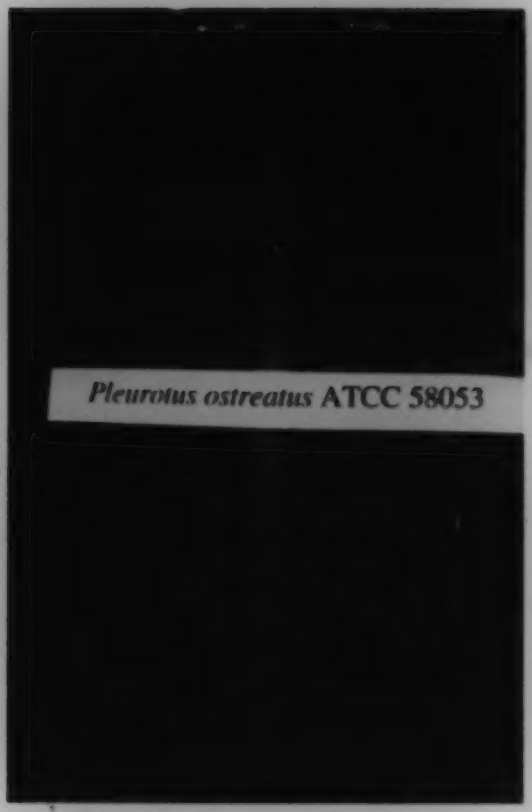
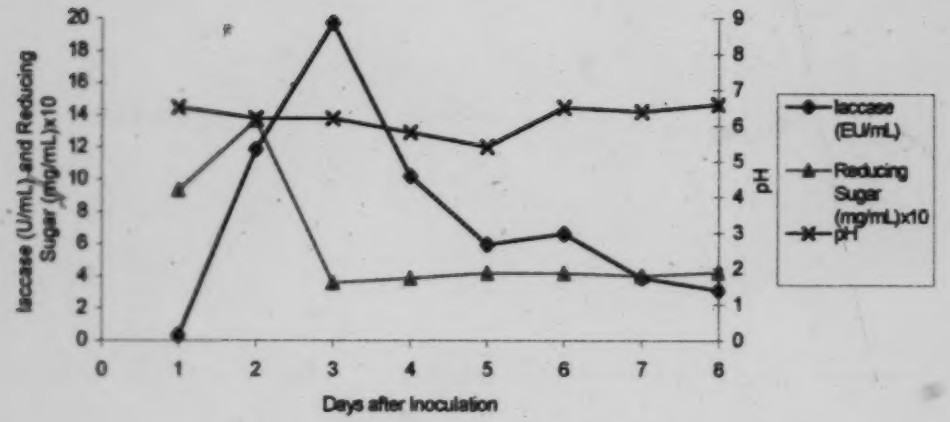
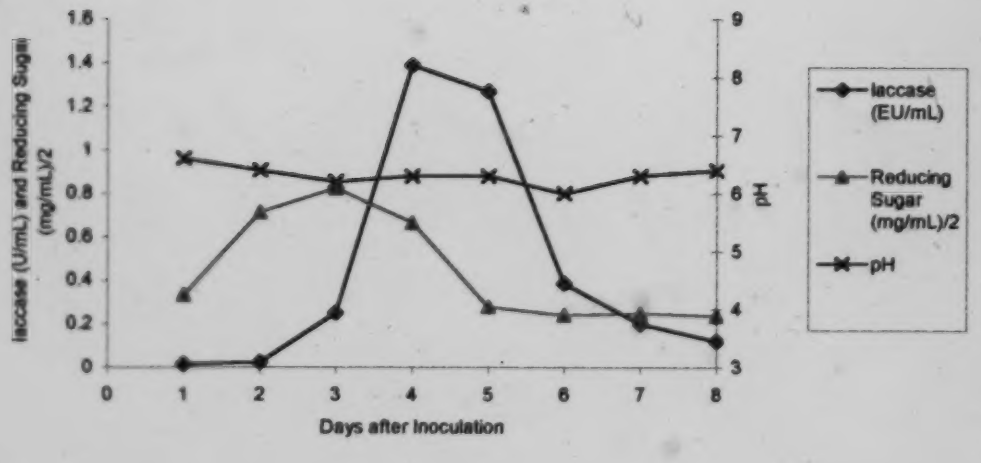


Figure 17: Fermenter at day three of incubation after inoculation with *Pleurotus ostreatus* ATCC 58053 (fourth trial protocol)



(A)



(B)

Figure 18: Fermenter production curves for *P. ostreatus* ATCC 58053 **(A)** and for *P. ostreatus* UAMH 7988 **(B)**

The method elucidated in the fourth trial was repeated with separate inoculations of *P. ostreatus* ATCC 58053 and UAMH 7988. In addition to laccase production, pH of the medium and reducing sugar content were also followed. These production curves are illustrated in Figure 18. Both curves describe similar trends, with laccase production increasing after reducing sugar values decrease, and with pH of the medium remaining stable at 6.0. These fermentations were repeated to produce enzyme for purification; this repeat generated 5.4 U/mL for UAMH 7988 and 16.5 U/mL for ATCC 58053; yielding a total of 43 200 total enzyme units and 133 650 total enzyme units respectively.

3.2 Purification and Characterization of Laccases

Purification of fungal laccases is simplified by the fact that they are secreted extracellularly: there is no need to liberate the enzyme from the cell along with many contaminating proteins. However, fungi also secrete a number of other proteins (although far less than the amount found intracellularly) and the medium used in this study also contains a large number of proteins. Further, fermentations with *P. ostreatus* ATCC 58053 and UAMH 7988 resulted in the production of a significant amount of pigment that imparted a deep brown colour to the clarified supernatant of these cultures. These pigments absorbed strongly in the ultraviolet range and appeared to be of a wide range of molecular weights and charges; the latter point makes their efficient removal especially troublesome. Accordingly, an extensive purification protocol was required and is described here.

Characterization of the purified laccases using a group of traditional measures of activity and structural characteristics was required to identify more precisely the enzymes isolated and to generate information useful in both the modification processes and

application to the degradation of PAHs. The results of several standard characterization techniques are described here.

3.2.1 Purification of Enzymes

Purification of laccase from the culture supernatants of *P. ostreatus* ATCC 58053 and UAMH 7988 followed the protocol as outlined in Methods and Materials (§ 2.5). Table 8 and Table 9 outline the relative success of these purifications for ATCC 58053 and UAMH 7988 respectively. The idiosyncrasies of the procedure and attendant qualitative observations for both purifications were essentially identical.

After removal of fungal biomass and unutilized bran, the culture supernatants were frozen, thawed and centrifuged; this facilitated the removal of the bulk of extracellular polysaccharides produced by these fungi. Only approximately half of each fermenter volume was purified at a single time; processing of the entire 8 L volume was prohibitive as once this volume was concentrated, the levels of pigment became too high to be easily removed. This supernatant was concentrated via ultrafiltration and while some of the lower molecular weight pigment passed through the 10 kDa cutoff used, most of the pigments were retained, producing an extremely dark solution.

Removal of these pigments could not be satisfactorily achieved using column separations alone. To alleviate this situation, polyethyleneimine (PEI) was added in a batchwise fashion to the ultrafiltration concentrate. In this way, PEI acted as an anionic exchange resin selectively removing pigment from enzyme at or below 0.2% (w/v). At 0.2% (w/v) a significant portion of the pigments were bound and the PEI : contaminant complexes were of high molecular weight and could be easily removed by centrifugation.

Table 8: Purification table for *P. ostreatus* ATCC 58053

[illegible]	Volume (mL)	Activity (U/mL)	Specific Activity (U/mg)	Purification (fold)	Yield (percent)
1- Thawed culture supernatant	4000	16.5	2.5	1	100
2- Ultrafiltration concentrate	300	210	3.5	1.4	95
3- PEI supernatant	290	207	6.0	2.4	91
4- Large DE-52	10	5550	20.2	8.1	84
5- Small DE-52	10	5235	42.5	17.0	79
6- Sephadex	5	9700	58.0	23.2	73
7- FPLC	5	9060	**61.0**	**24.4**	**69**

Table 9: Purification table for *P. ostreatus* UAMH 7988

[illegible]	Volume (mL)	Activity (U/mL)	Specific Activity (U/mg)	Purification (fold)	Yield (percent)
1- Thawed culture supernatant	3500	5.4	4.1	1	100
2- Ultrafiltration concentrate	250	72	6.5	1.6	95
3- PEI supernatant	245	69	10.8	2.6	90
4- Large DE-52	8	1875	37.8	9.2	80
5- Small DE-52	8	1650	75.0	18.2	70
6- Sephadex	5	2355	92.3	22.5	62
7- FPLC	5	2050	**95.0**	**23.2**	**54**

After this point, further purification was achieved with traditional anion exchange (DE-52), size exclusion (Sephadex) and FPLC chromatography.

P. ostreatus ATCC 58053 laccase was purified 24.2 fold with a final yield of 69%; the numbers for UAMH 7988 are similar at 23.2 fold and 54%. Each chromatographic step up to and including Sephadex size exclusion for both proteins resulted in a significant increase in specific activity. The final chromatographic step, FPLC, yielded only a small and probably non-significant increase in specific activity, indicating that both preparations were most likely free of contamination before this point. Preparations from FPLC had a brilliant blue colour, indicative of a concentrated, pure preparation of copper-containing laccase. The final specific activity of *P. ostreatus* UAMH 7988 was approximately 50% higher (95.0 U/mg) than the specific activity for *P. ostreatus* ATCC 58053 (61.0 U/mg) based on ABTS oxidation and the Bradford dye binding protein assay with bovine gamma globulin as the standard protein.

3.2.2 Characterization of Purified Laccases

3.2.2.1 Temperature Optima

Figure 19 illustrates the relative activity of both purified laccases at several temperatures. The temperature optimum displayed for UAMH 7988 is 20 °C lower (40 °C) than that for ATCC 58053 (60 °C). These values were obtained over 15 s immediately after the enzyme was added to pre-equilibrated reaction mixtures.

3.2.2.2 Temperature Stability

The temperature stabilities of each laccase at 4, 22 and 65 °C are illustrated in

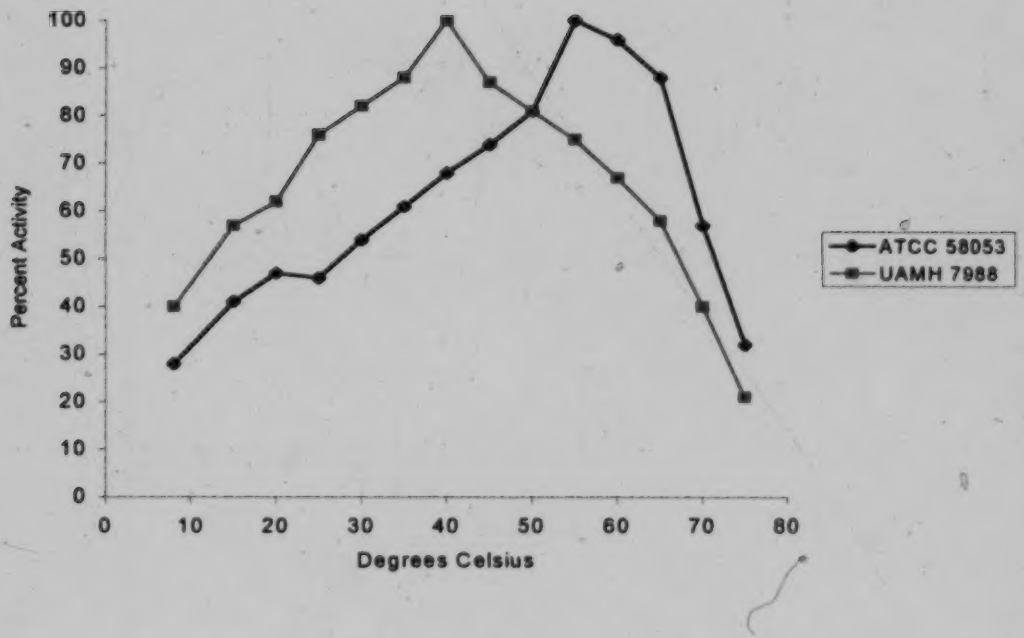


Figure 19: Temperature optima for laccases from *P. ostreatus* ATCC 58053 and UAMH 7988.

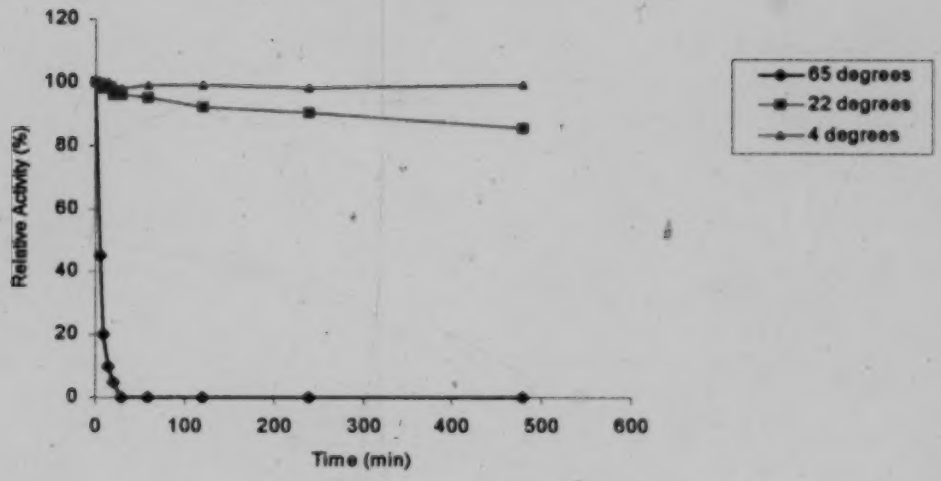


Figure 20: Temperature stability for *P. ostreatus* ATCC 58053 laccase

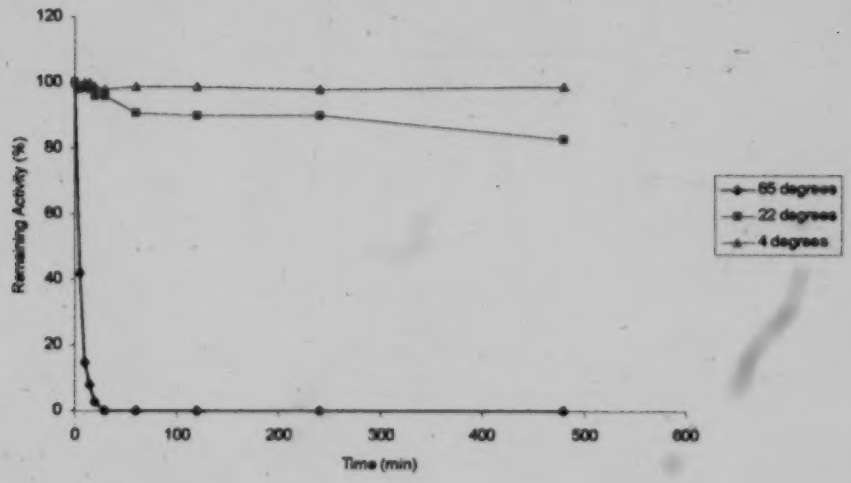


Figure 21: Temperature stability for *P. ostreatus* UAMH 7988 laccase

Figures 20 and 21 for ATCC 58053 and UAMH 7988 respectively. The two laccases illustrate essentially identical trends for each of the temperatures tested. The enzymes are extremely stable at 4 °C with half-lives well beyond the duration of the experiment for both ATCC 58053 and UAMH 7988. These enzymes show a similar pattern of stability at 25 °C, again with similar half-lives over the time tested. However, at 65 °C, both enzymes rapidly lose activity due to thermal denaturing, with 90% of their activity lost within 15 minutes.

3.2.2.3 pH Optima for Catalysis

Both of the laccases tested showed a distinct pH optimum for catalysis at 3.0 with ABTS as the oxidized substrate, as illustrated in Figure 22. These laccases also demonstrated a propensity for acidic pH optima with the other substrates tested later during kinetic studies. Succeedingly more alkaline reaction mixtures produce a drop in activity, however, both enzymes retain nearly 50% of their activity at pH 5.0.

3.2.2.4 pH Stability

Figure 23 and Figure 24 demonstrate the stability of each of these laccases at a variety of different pH values. Each of these enzymes is most stable at pH 9; succeedingly more acidic incubation pH values also lead to greater losses in activity. The laccase isolated from ATCC 58053 demonstrates less of a drop in activity than is seen with UAMH 7988 at pH 8; in fact, this laccase retained marginally more activity with each of the more acidic pH values tested. These laccases are significantly less stable at their optimal pH values for catalysis than more alkaline pH values.

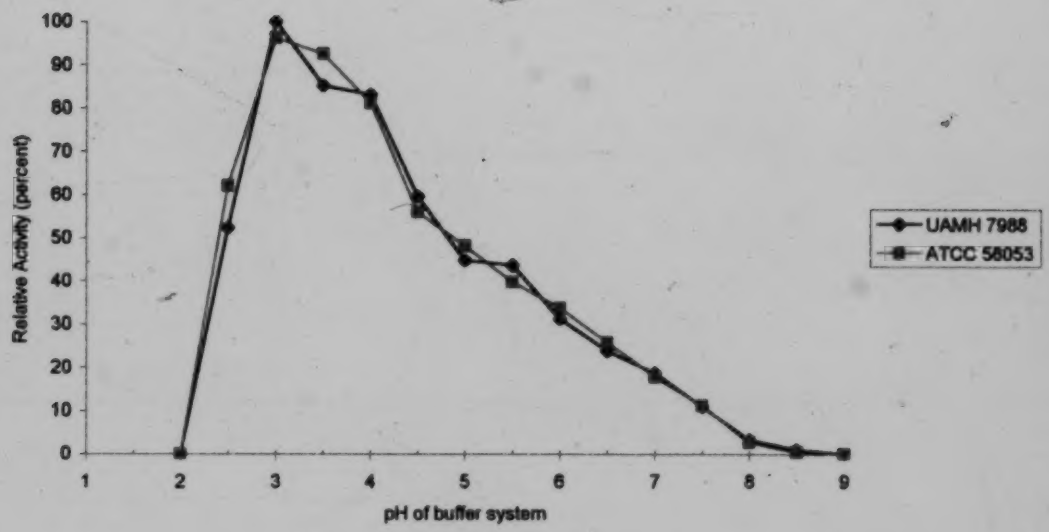


Figure 22: pH optima profiles of *Pleurotus ostreatus* ATCC 58053 and UAMH 7988 laccases

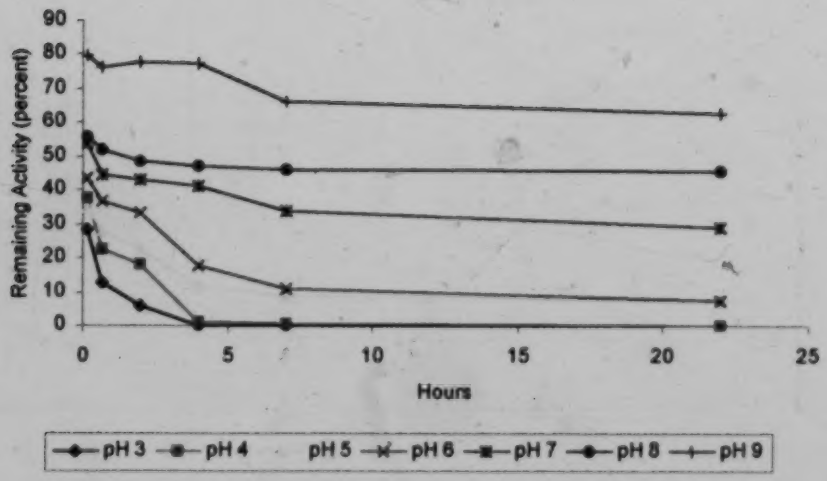


Figure 23: pH stability of laccase from *Pleurotus ostreatus* ATCC 58053

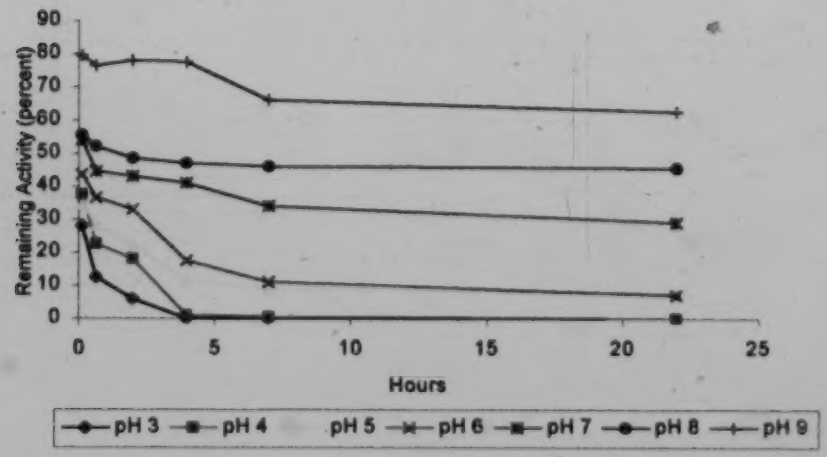


Figure 24: pH stability of laccase from *Pleurotus ostreatus* UAMH 7988

3.2.2.5 Polyacrylamide Gel Electrophoresis

The laccase preparations were further analyzed using gel electrophoresis. Figure 25 displays the results of a polyacrylamide gel in which both denatured and native forms of the laccase preparations were run with later total protein and activity staining. The denatured preparations (lanes 2 and 3) for both ATCC 58053 and UAMH 7988 ran to the same position on the gel, ca. 60 kDa as measured by reference with the molecular weight standards in lanes 1 and 6. Interestingly, native forms of the laccases (lanes 4, 5, 7 and 8) ran a significant distance further in the gel, to approximately 34 kDa, again measured in reference to the standards in lanes 1 and 6. This demonstrates that these proteins likely have a significantly compact shape in their native conformation. The electrophoresis of native samples also allowed an activity stain to be performed. Lanes 7 and 8 demonstrate the results of this activity stain, and illustrate the homogeneity of the preparation with one clear band for each sample. Some microheterogeneity can be observed in the total protein staining samples.

3.2.2.6 Molecular Weight by Mass Spectrometry

Mass spectrometric analysis of the laccase preparations was also performed using matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) spectrometry to provide a more accurate estimation of molecular weight. The molecular weights obtained from lanes 4 and 5 of Figure 25, and in general most molecular weights obtained from polyacrylamide gel electrophoresis, are somewhat suspect because of the number of variables that can influence migration through the gel besides the absolute molecular weight of the protein. The results of the more accurate MALDI-TOF analysis for the laccase preparations from ATCC 58053 and UAMH 7988 can be seen in Figure 26. Each preparation gave a

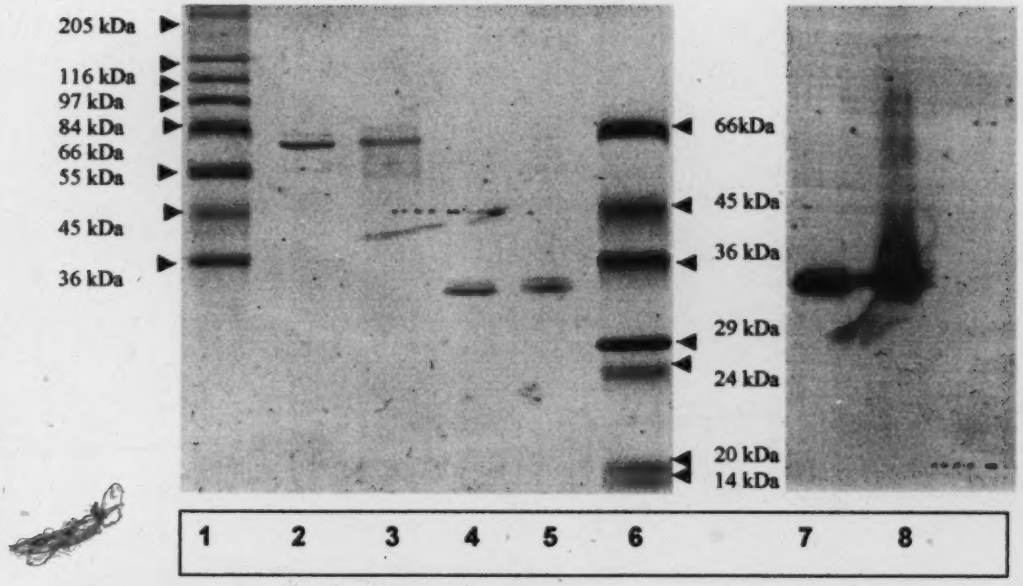


Figure 25: Polyacrylamide gel electrophoresis of *P. ostreatus* ATCC 58053 and UAMH 7988 laccases under denaturing (lanes 2 & 3) and non-denaturing (lanes 4 & 5 and 7 & 8) conditions with protein (lane 1-6) and activity (lanes 7 and 8) staining.

Lane 1: High Molecular Weight Markers

Lane 2: 5 µg *P. ostreatus* ATCC 58053 laccase; boiled 5 minutes in 5% β-mercaptoethanol

Lane 3: 5 µg *P. ostreatus* UAMH 7988 laccase; boiled 5 minutes in 5% β-mercaptoethanol

Lane 4: 5 µg *P. ostreatus* ATCC 58053 laccase; undenatured

Lane 5: 5 µg *P. ostreatus* UAMH 7988 laccase; undenatured

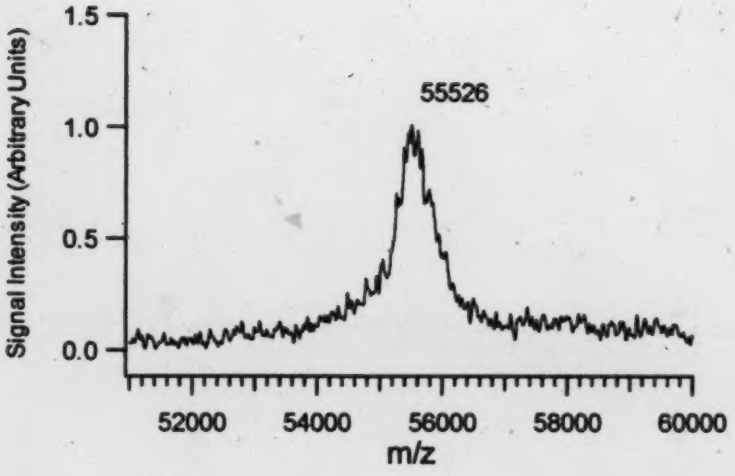
Lane 6: Low Molecular Weight Markers

Lanes 1-6 were stained for total protein using Comassie blue.

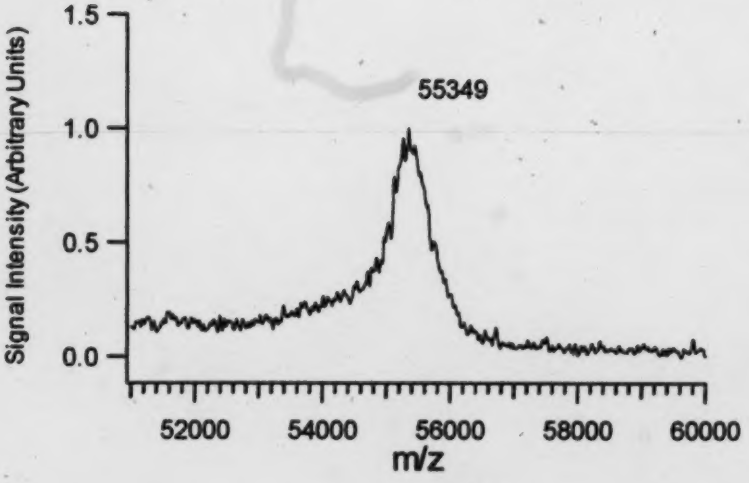
Lane 7: 5 µg *P. ostreatus* ATCC 58053 laccase; undenatured

Lane 8: 5 µg *P. ostreatus* UAMH 7988 laccase; undenatured

Lanes 7 and 8 were stained for laccase activity with a 1 mM solution of o-dianisidine in 10% methanol.



(A)



(B)

Figure 26: MALDI-TOF analysis of molecular weight for *P. ostreatus* ATCC 58053 laccase (signal intensity peaks at a molecular weight of 55 526 Da)**(A)** and *P. ostreatus* UAMH 7988 laccase (signal intensity peaks at a molecular weight of 55 349)**(B)**

single clear peak in the uncharged range, further indicating the purity of the preparation. Both values obtained for molecular weight are similar, with a final value of 55 526 Da for *P. ostreatus* ATCC 58053 and 55 349 Da for *P. ostreatus* UAMH 7988. These values are approximately 7.5 % less than the value of 60 kDa for each obtained from polyacrylamide gel electrophoresis.

3.2.2.7 Isoelectric Focussing

The laccase preparations were also subjected to isoelectric focussing as part of their further characterization. The results of this work can be seen in Figure 27; both laccase preparations again ran to the same position in the gel, indicating an identical isoelectric point (IeP) for both. The calculated IePs for both laccases are 3.4, inferred from a standard curve drawn from the migration of the other standard proteins in the gel. Both preparations nearly ran off the gel and are below the scope of most commercially prepared standards. Chloroperoxidase from *Caldariomyces fumago*, which itself has an acidic IeP (3.4), was available as a result of earlier work in this laboratory (Pickard and Hashimoto, 1988). The nature of the bands seen in this isoelectric focussing is also important. Each preparation displays a single, clear band; this information, coupled with the results seen in earlier sections, strongly suggests that the laccase preparations are pure and contain only a single isoenzyme.

3.2.2.8 Spectra of Laccase Preparations and Copper Content Analysis

Figures 28 and 29 illustrate the ultraviolet-visible spectrum of the laccase preparations from *P. ostreatus* ATCC 58053 and UAMH 7988 respectively. Both spectra are typical of laccases in general, and have several important characteristics. A slight shoulder is seen in both cases at 330 nm, and this is indicative of the presence of a type 3 copper center;

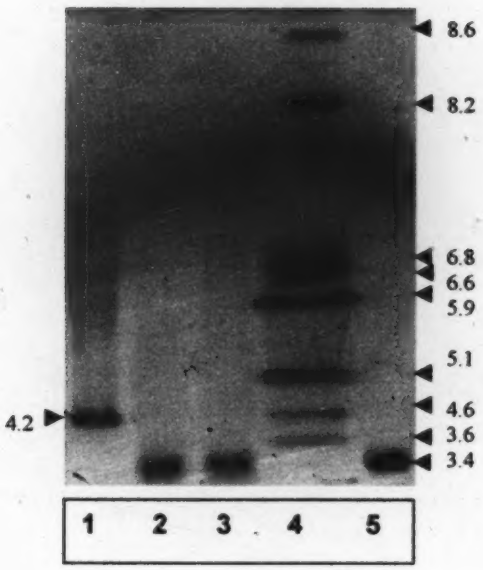


Figure 27: Isoelectric focussing of *P. ostreatus* ATCC 58053 and UAMH 7988 laccases.

Lane 1: Glucose oxidase, *Aspergillus niger*	pI = 4.2
Lane 2: *Pleurotus ostreatus* ATCC 58053 laccase calculated	pI = 3.4
Lane 3: *Pleurotus ostreatus* ATCC 58053 laccase calculated	pI = 3.4
Lane 4: Range of IEF Standards	
Lane 5: Chloroperoxidase, *Caldarimyces fumago*	pI = 3.4

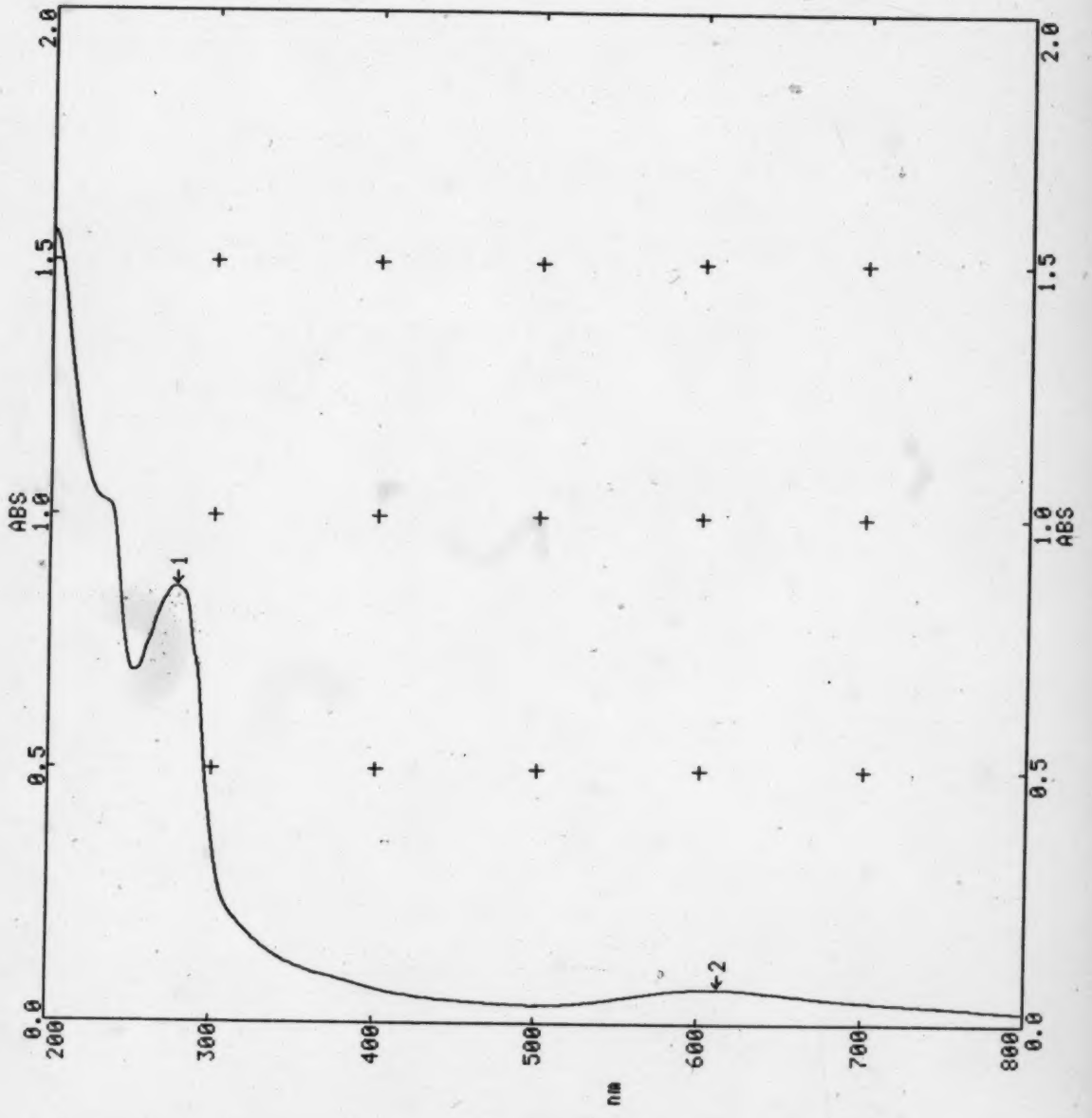


Figure 28: UV-Visible spectrum of native *P. ostreatus* ATCC 58053 laccase

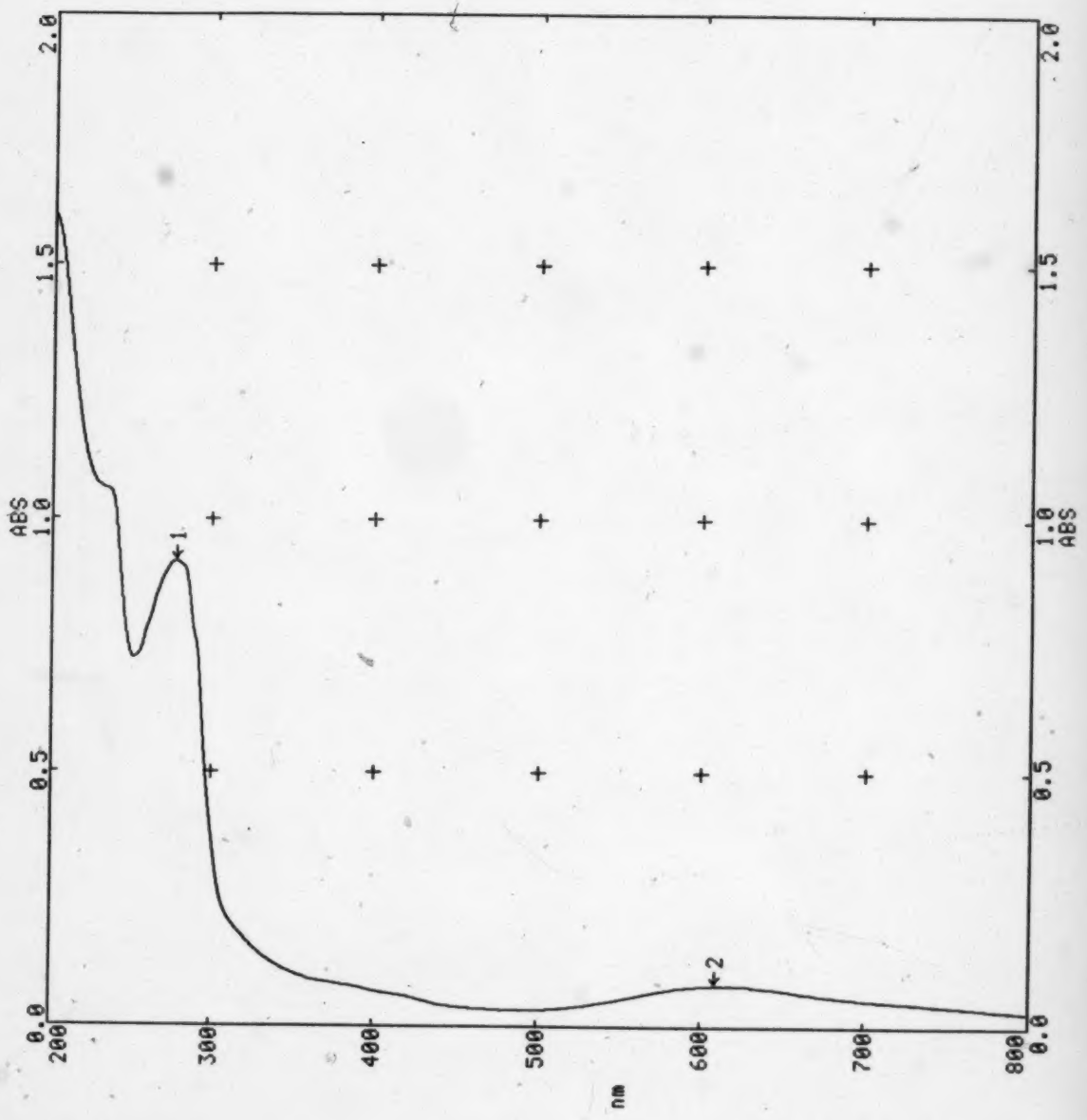


Figure 29: UV-Visible spectrum of native *P. ostreatus* UAMH 7988 laccase

further, another slight peak at 610 nm demonstrates the presence of a type 1 copper center (Korolijova *et al*, 1999). It is this type 1 copper absorbing in the visible range that imparts the purified laccase preparations with a deep blue colour. Analysis of the total copper content of these proteins demonstrated the presence of 3.6 and 3.8 Cu atoms per enzyme for *P. ostreatus* ATCC 58053 and UAMH 7988 respectively. These values and the information from the spectra indicate that in all likelihood, these two laccases have the typical complement of four copper atoms (two type 3, one type 1 and one type 2 copper centers) per protein molecule.

Another indicator of the purity of these preparations can be found in observing the A_{280} to A_{610} ratios seen in their spectra. These values are 11.9 and 10.9 for ATCC 58053 and UAMH 7988 respectively.

3.2.2.9 Carbohydrate Content

Laccases, like most secreted fungal proteins, are typically glycosylated. A total carbohydrate analysis indicated that 15.2 % and 18.4 % of the molecular weights of ATCC 58053 and UAMH 7988 laccases were composed of linked carbohydrate moieties, respectively.

3.2.2.10 N-Terminal Sequences and Amino Acid Content

The N-terminal sequences of the two laccases were also determined and are presented in Table 10. Included in Table 10 for comparative purposes are a number of other N-terminal sequences of laccases isolated from different sources. Table 11 illustrates the results of an amino acid analysis profile performed on the laccase from ATCC 58053. The

Table 10: N-Terminal sequences of *P. ostreatus* ATCC 58053 and UAMH 7988 laccases

Organism	Sequence
P. ostreatus ATCC 58053	A I G P A G N M Y I V N E D V S P D G F
P. ostreatus UAMH 7988	A I G P A G N M Y I V N E D V S P D G F
Coriolopsis gallica UAMH 8260	**A I G P** V A D L T **I** S **N** G A **V S P D G F**
Trametes versicolor	G **I G P** V A D L T **I** N A A V S P D G F
Coriolis hirsutus	**A I G P** T A D L T **I** S **N** A E **V S P D G F**
Phelbia radiata	S **I G P** V T D F H **I V N** A A **V S P D G F**
Pycnoporus cinnabarinus	**A I G P** V A D L T L T **N** A A **V S P D G F**
Neurospora crassa	G G **G** G G C **N** S P T N R O C W S **P**
Coprinus friesii	**A I G P** V A D L **Y I** G **N** K V I A **P D G F**
Ceriporiopsis subvermispora	**A I G P** V T D I E **I** T D A F **V S P D G** P

Bold letters indicate common residues

Table 11: Partial amino acid composition profile of *P. ostreatus* ATCC 58053 laccase

Amino Acid	Percent*
Aspartic acid	11.2
Serine	7.2
Proline	6.8
Alanine	9.4
Valine	7.0
Isoleucine	4.4
Tyrosine	2.8
Histidine	3.0
Arginine	3.8
Threonine	5.6
Glutamic acid	10.6
Glycine and Cysteine	9.4
Methionine	1.0
Leucine	8.4
Phenylalanine	4.4
Lysine	5.0

*: Calculation excludes contributions from asparagine, glutamine and tryptophan as these amino acids cannot be detected via the method used.

Table 12: Properties of laccase from *P. ostreatus* (strain Florida) coded by *pox2*

```
    1          11         21         31         41         51
  1 MFPGARILAT LTLALHLLHG AHAAIGPAGN MYIVNEDVSP DGFARSAVVA RSVPATDPTP   60
 61 ATASIPGVLV QGNKGDNFQL NVVNQLSDTT MLKTTSIHWH GFFQAGSSWA DGPAFVTQCP  120
121 VASGDSFLYN FNVPDQAGTF WYHSHLSTQY CDGLRGPFVV YDPSDPHLSL YDIDNADTVI  180
181 TLEDWYHIVA PQNAAIPTPD STLINGKGRY AGGPTSPLAI INVESNKRYR FRLVSMSCDP  240
241 NFTFSIDGHS LLVIEADAVN IVPITVDSIQ IFAGQRYSFV LTANQAVDNY WIRANPNLGS  300
301 TGFVGGINSA ILRYAGAIED DPTTTSSTST PLLETNLVPL ENPGAPGPPV PGGADININL  360
361 AMAFDFTTFE LTINGVPFLP PTAPVLLQIL SGASTAASLL PSGSIYELEA NKVVEISMPA  420
421 LAVGGPHPFH LHGHTFDVIR SAGSTTYNFD TPARRDVVNT GTGANDNVTI RFVTDNPGPW  480
481 FLHCHIDWHL EIGLAVVFAE DVTSISAPPA AWDDLCPIYN ALSDNDKGGI VPS
```

23 amino acid leader (in black), 510 amino acid expressed chain (in red)
N-terminal sequence of mature protein underlined in blue
Calculated pI from amino acid sequence = 4.51

Calculated molecular weight from amino acid sequence = 54 360

Putative copper binding residues at 98, 100, 143, 145, 427, 430, 432, 483, 484, 485, 489

N-glycosylated at Asn-467 by a high mannose structure; the number of attached mannoses can vary substantially

Sequence information from Giardina *et al.* (1996)

N-terminal sequences of *P. ostreatus* ATCC 58053 and UAMH 7988 laccases are identical, further highlighting the similarity between these two enzymes. The N-terminal sequences of these two laccases are in turn also identical to another from a different strain of *P. ostreatus* not displayed in Table 10. This sequence can be found in Table 12, along with the other elucidated characteristics of this laccase from *P. ostreatus* (strain Florida). The similarities between the laccases produced in this study and the laccase from *P. ostreatus* (strain Florida) indicate that there is a high degree of probability that the entire amino acid sequences of all three are very similar.

3.3 Modification of Laccases and Application in PAH Oxidation

After characterization, the purified laccases were subjected to modification via the attachment of the monofunctional polymers PEG and Brij35; the first subsection here describes the success of the procedure used in attaching these groups. After modification, the enzymes were then subjected to characterization to determine the effect, if any, of these procedures on their catalytic ability and specificity. Further, the impact of chemical modification on catalytic ability in a number of organic solvents was also examined; the second section presents these results. Lastly, the modified and unmodified enzymes were utilized in a system containing organic solvent to oxidized various PAHs; these results are presented in the final section of these results.

3.3.1 Chemical Modification

3.3.1.1 Method and Extent of Modification

Chemical modification of the two laccases in question was performed by covalently attaching two different monofunctional polymers to available amino groups on the surface of the protein. The procedures utilized make use of alkaline environments and are outlined

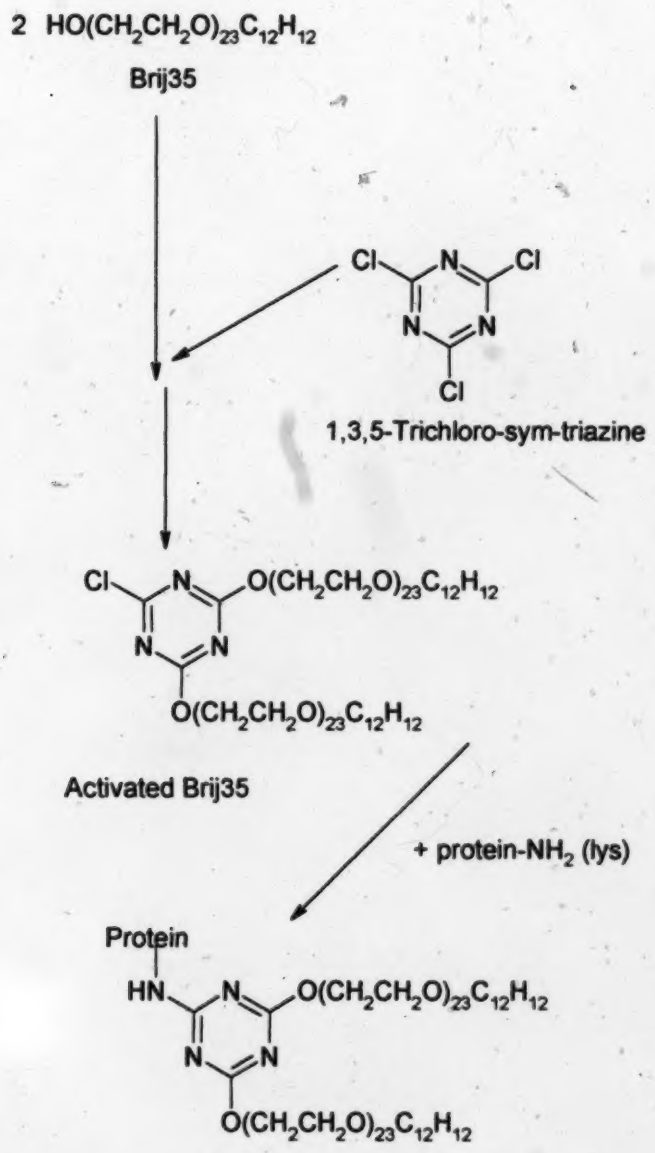


Figure 30: Brij35 activation and reaction at amino groups on protein surface

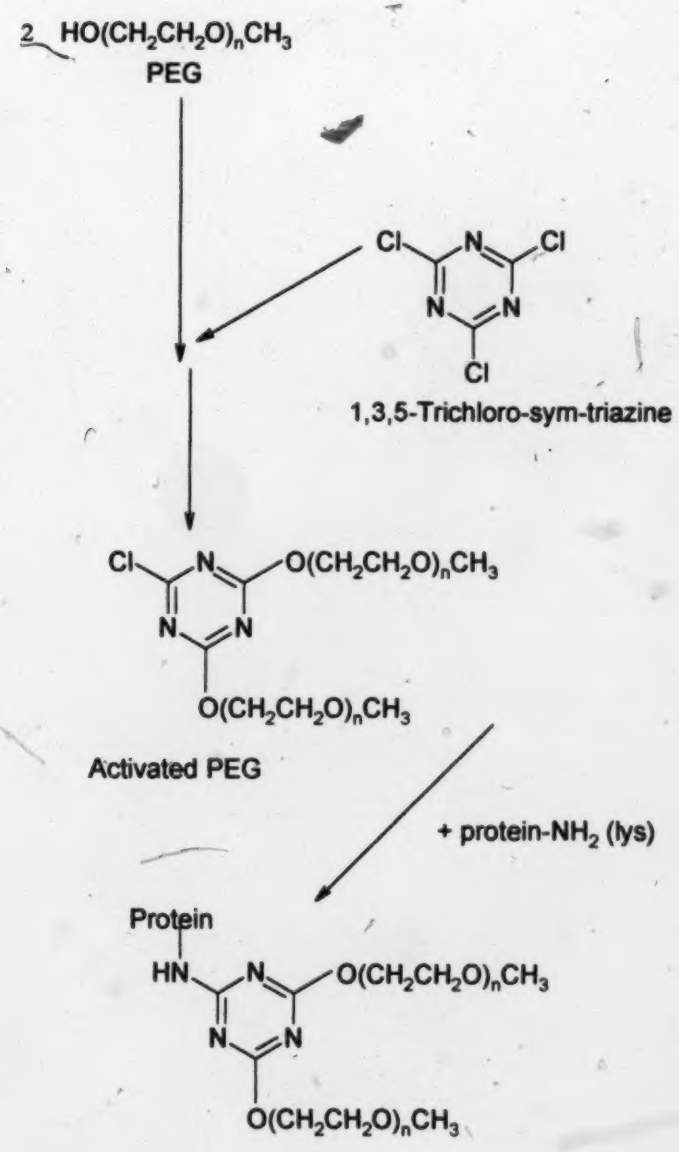


Figure 31: Polyethylene glycol (PEG) activation and reaction at amino groups on protein surface

in Methods and Materials. Figure 30 and Figure 31 demonstrate the process of the addition of these two groups.

The extent of chemical modification of the enzymes was measured by determining the relative amount of free amino groups per enzyme before and after the procedure in an assay utilizing trinitrobenzosulfonic acid. The results of this assay indicated that 34 % and 45 % of the free amino groups on *P. ostreatus* ATCC 58053 and UAMH 7988 were modified respectively.

3.3.1.2 Spectra of Modified Enzymes

Figure 32 and Figure 33 illustrate the ultraviolet-visible spectra of *P. ostreatus* ATCC 58053 and UAMH 7988 laccases after modification with Brij 35, respectively. Spectra obtained after modification of these enzymes with PEG were very similar. In each case, absorbance at 610 nm is depressed with respect to the unmodified enzyme and there is a significant broadening of the peak seen at around 280 nm. Both Brij 35 and PEG absorb strongly in the ultraviolet; their binding to the enzyme is most likely responsible for the differences at around 280 nm. However, the loss of absorbance at 610 nm may be indicative of a change in conformation of the enzyme and in particular at the type 1 copper center responsible for electron capture.

3.3.1.3 Characteristics after Modification

The pH optima and stability as well as the temperature optima and stability of the two enzymes were identical to those of the native enzyme after each modification. Further, all volumetric activity was recovered after the modification procedure (with ABTS as the

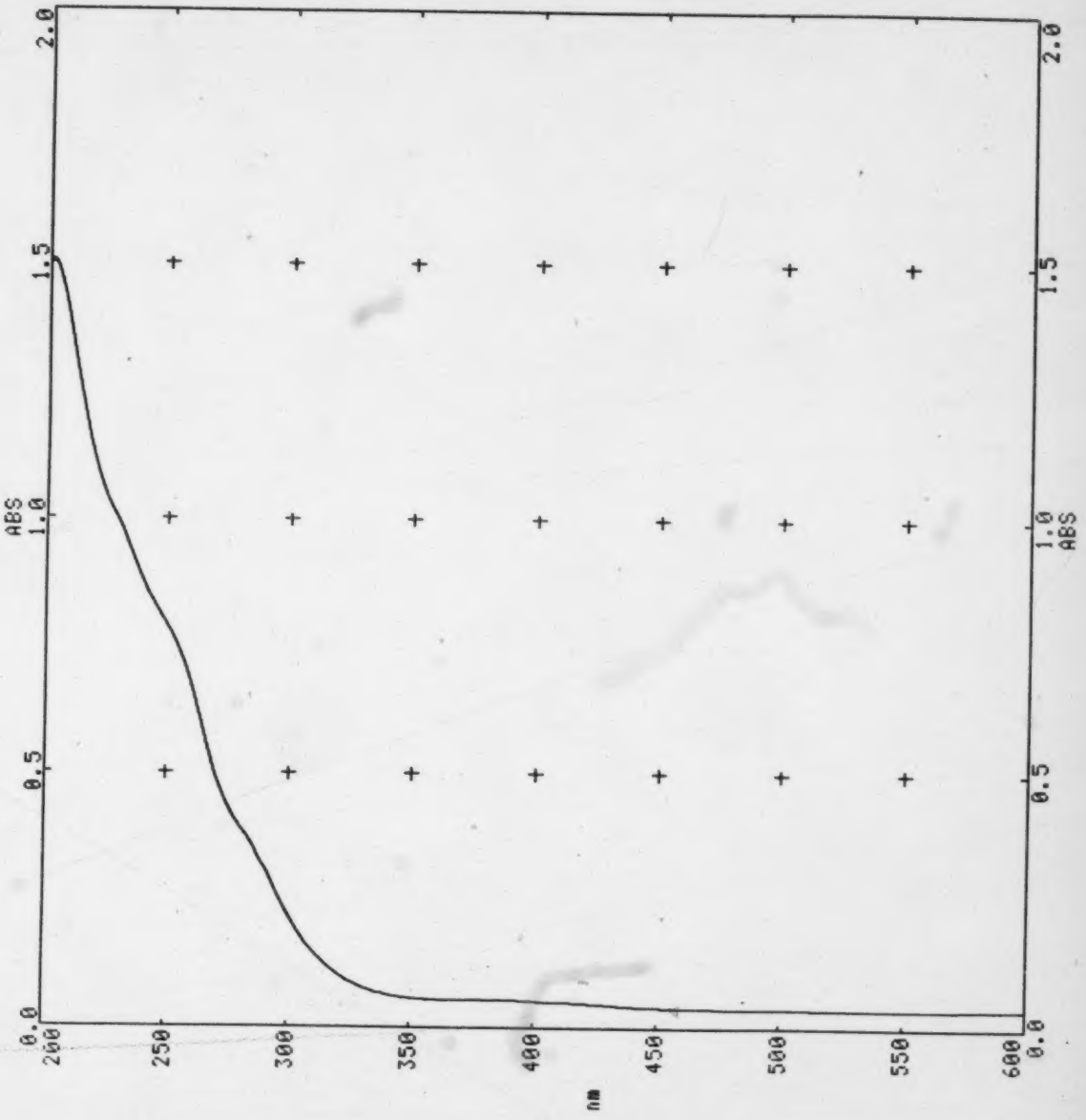


Figure 32: UV-Vis spectra of *P. ostreatus* ATCC 58053 laccase modified with Brij35

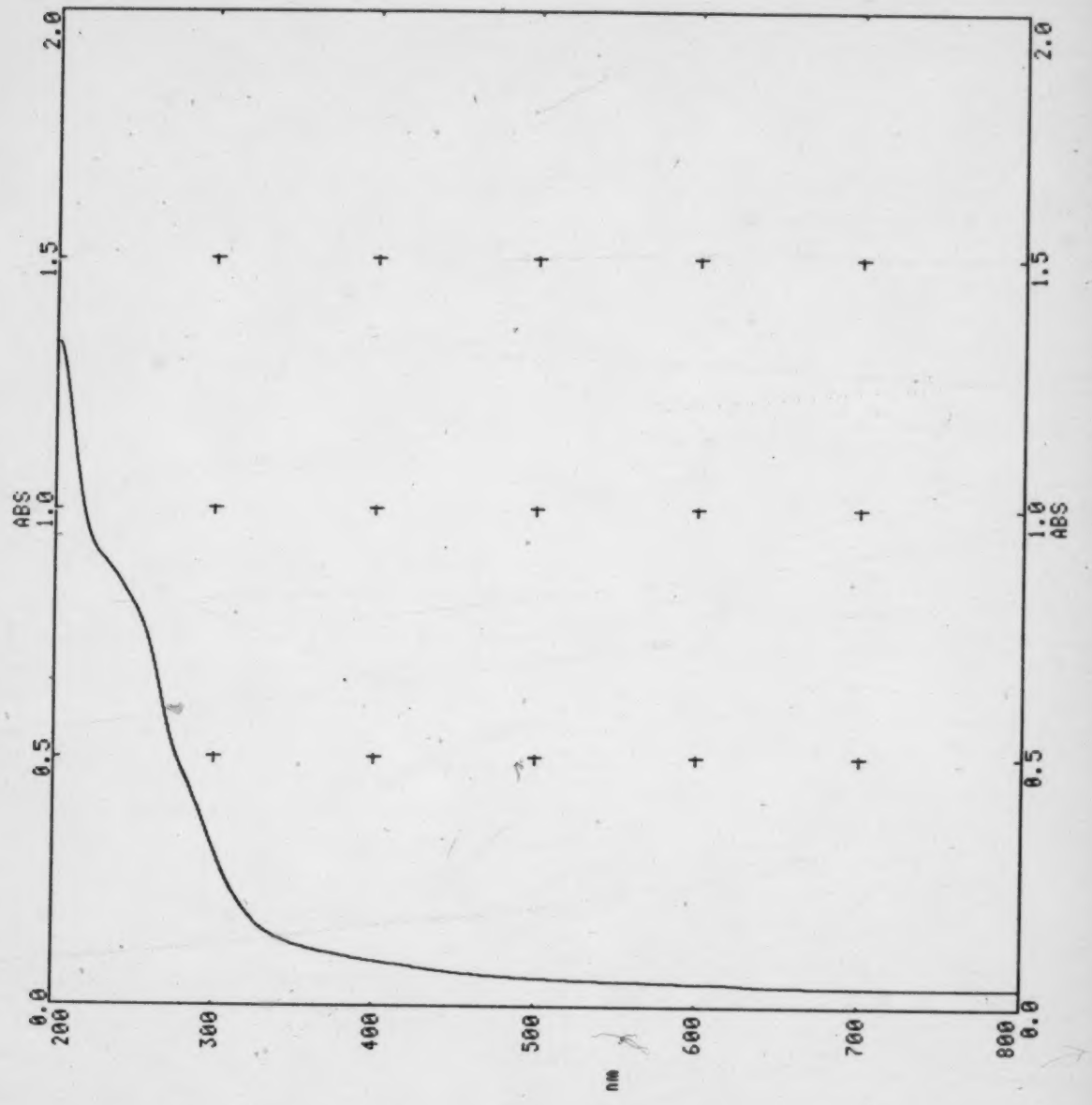


Figure 33: UV-Vis spectra of *P.ostreatus* UAMH 7988 laccase modified with Brij35

substrate). Volumetric tracking of the total amount of protein was required as traditional assays of protein concentration could be expected to be interfered with by the binding of the polymers to the positively charged amino groups, which are often contributors to a positive response in most protein assays.

3.3.1.4 Kinetics Before and After Modification

The K_m for several substrates was measured with both the modified and unmodified forms of each laccase. The results of this analysis can be seen in Table 13. With 2,6-dimethoxyphenol, the K_m is seen to drop by around a factor of ten for each modification compared with the native laccase for both *P. ostreatus* ATCC 58053 and UAMH 7988. However, the modified enzymes display similar K_m values as their native counterparts for the remaining four substrates tested. Overall, the results indicate that the catalytic abilities of the isolated laccases were not drastically affected by the modification procedure.

3.3.2 Organic Solvents and Enzyme Catalysis

3.3.2.1 Enzyme Stability in Organic Solvents

Each of the modified and unmodified enzymes were tested for their ability to retain activity after exposure to different concentrations of organic solvents. Each displayed half-lives that were inversely proportional to the concentration of organic solvent in the incubation mixture. These experiments were carried out over a range of solvent concentrations but Table 14 demonstrates the results of just one set of these incubations. All of the modifications attempted were successful in increasing the half-life of each enzyme in acetonitrile. Conversely, the modifications appeared to be of minimal or no benefit in

Table 13: K_m values for modified and unmodified *P. ostreatus* ATCC 58053 and UAMH 7988 laccases with a number of substrates

	P. ostreatus ATCC 58053 laccase K_m (mM)			*P. ostreatus* UAMH 7988 laccase K_m (mM)		
Substrate	Native	PEG	Big55	Native	PEG	Big55
ABTS	0.19	0.093	0.046	0.048	0.071	0.085
2,6-Dimethoxyphenol	1.26	0.11	0.12	2.22	0.26	0.13
Catechol	6.52	5.84	5.23	5.10	5.85	5.52
Guaiacol	3.32	3.85	2.55	5.16	5.74	5.91
4-Aminophenol	2.01	1.00	1.18	0.0048	0.023	0.039

Table 14: Half-lives of modified and unmodified laccases in organic solvents

	Half-life of laccase from *P. ostreatus* ATCC 58053 (min)			Half-life of laccase from *P. ostreatus* ATCC 58053 (min)		
Organic Solvent	Native	PEG	Brij35	Native	PEG	Brij35
Acetonitrile (20%)	2.0	6.0	14.8	1.3	3.2	7.0
Dimethylsulfoxide (50%)	28.6	72.5	75.3	17.1	17.9	16.8
N,N'-dimethylformamide (40%)	19.5	23.2	21.5	9.6	7.2	8.5

increasing the half-lives of these enzymes in N,N'-dimethylformamide. The results regarding dimethylsulfoxide were mixed; the modifications appear very successful at increasing half-life with *P. ostreatus* ATCC 58053 laccase but do not have any effect with *P. ostreatus* UAMH 7988 laccase.

3.3.2.2 Laccase Activity in Organic Solvents

The effect of increasing amounts of organic solvent on the kinetic constants of each form of the enzymes was also investigated. The relative K_{cat} of each enzyme was followed in reaction systems utilizing ABTS as the substrate (because of its high solubility) and increasing concentrations of organic solvent. In each case, an increase in organic solvent led to dramatic and immediate increases in the K_m of each enzyme for ABTS, necessitating the use of a wide range of substrate concentrations to ascertain an accurate value for V_{max} at each point.

Figure 34 and Figure 35 demonstrate the effect of increasing concentrations of acetonitrile (CH_3CN) on the catalytic ability of *P. ostreatus* ATCC 58053 and UAMH 7988 modified and unmodified laccases respectively. In Figure 34, the activity of the native laccase can be seen to drop off sharply after 20 % CH_3CN, reaching zero at 60 %. The concentration at which ½ activity is reached by the line of best fit (see appendix for fitted equation) or EC_{50}, is 29.3 %. The EC_{50} values for the PEG and Brij laccases in this case are considerably higher, at 43.7 % and 34.4 % respectively. However, the EC_{50} value does not take into account the slope of the line seen with the Brij laccase; it alone is only a good measure of the increase in protection if the slopes of the modified and unmodified lines are closely similar, as with the native and PEG laccases in this case. More illustrative of the beneficial effect of the Brij modification here is the greater activity seen at 30 % CH_3CN

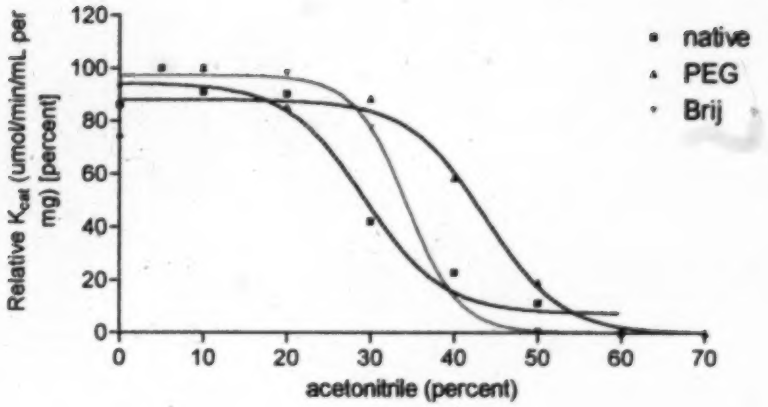


Figure 34: Effect of acetonitrile on K_{cat} for *P. ostreatus* ATCC 58053 laccase

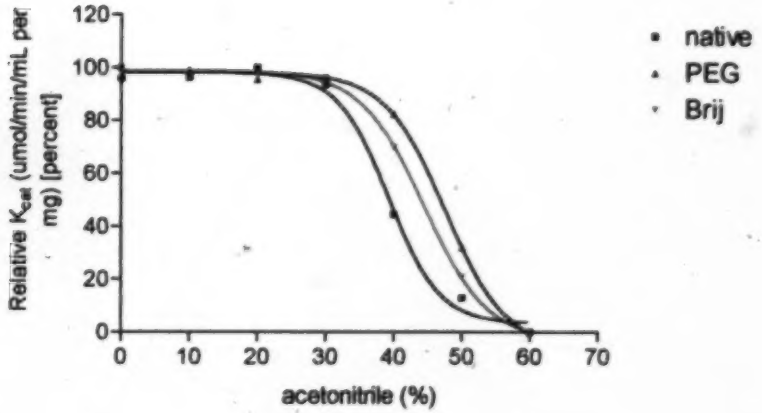


Figure 35: Effect of acetonitrile on K_{cat} for *Pleurotus ostreatus* UAMH 7988 laccase

concentration. Similar results are seen with the laccase from *P. ostreatus* UAMH 7988 in Figure 35, however, the results are less drastic. The native laccase exhibits an EC_{50} of 39.3 % CH_3CN, while the same values for the Brij and PEG modified laccases are 44.5 % and 47.6 % respectively.

Figure 36 and Figure 37 illustrate the effect of increasing amounts of dimethylsulfoxide (DMSO) on the catalytic activity of the enzymes from ATCC 58053 and UAMH 7988 repectively. Again, both figures describe similar trends with all laccases tested. In Figure 36, the EC_{50} value for the native laccase is 45.0 % DMSO while the EC_{50} values of the Brij and PEG modified enzymes are 51.1 % and 49.6 % DMSO respectively. In Figure 37, the EC_{50} value for the native laccase is 44.9 % DMSO whereas the EC_{50} values of the Brij and PEG modified enzymes are 47.7 % and 50.0 % DMSO respectively. However, it is prudent to note that in each case, the modified enzymes retain nearly 100 % of their activity at 40 % DMSO whereas the native enzymes have already experienced drastic reductions in activity at this concentration.

The effects of modification on retention of activity for the modified laccases is much less apparent in study with N,N'-dimethylformamide (DMF) than it was for the previous two solvents. In Figure 38, the relative activity of *P. ostreatus* ATCC 58053 native and modified laccases are also compared; little difference is apparent in the curves displayed. The EC_{50} value for the native laccase is 41.1 % DMF while the same values for the Brij and PEG modified laccases are 41.8 % and 44.3 % respectively. Again, a similar set of trends can be observed with the laccases from *P. ostreatus* UAMH 7988 in Figure 39. The EC_{50} value of the native laccase in this case is 40.7 % while the respective values for the Brij and PEG laccases are 40.1 % and 42.0 %.

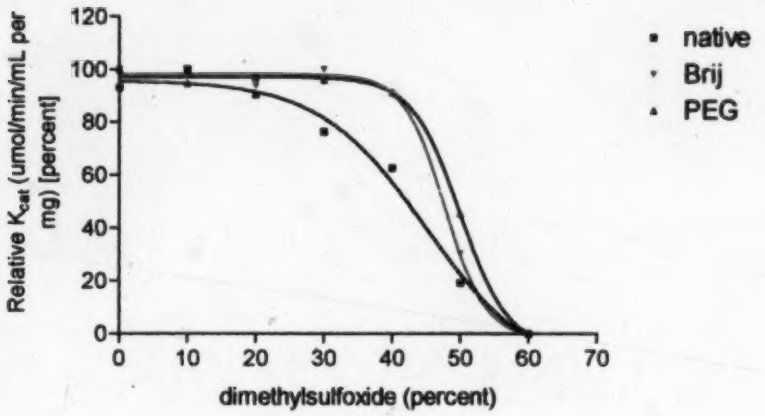


Figure 36: Effect of dimethylsulfoxide on K_{cat} for *P. ostreatus* ATCC 58053 laccase

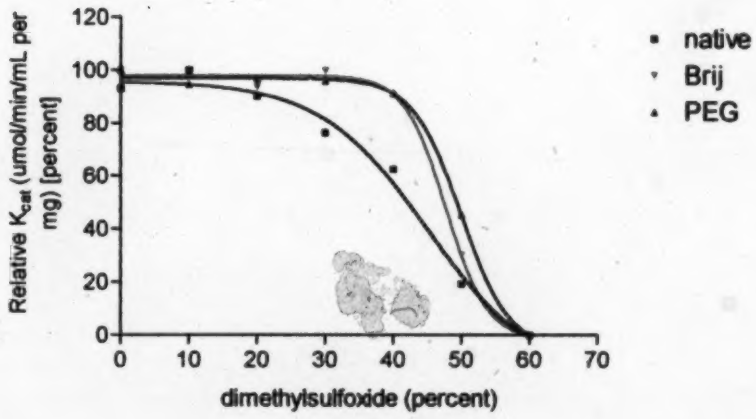


Figure 37: Effect of dimethylsulfoxide on K_{cat} for *P. ostreatus* UAMH 7988 laccase

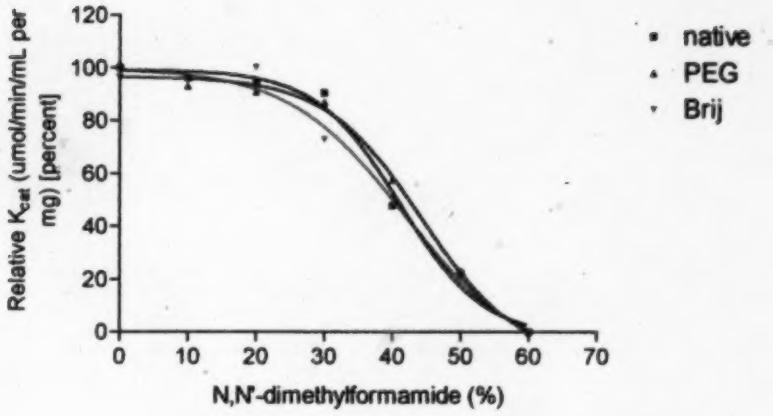


Figure 38: Effect of N,N'-dimethylformamide on K_{cat} for *P. ostreatus* ATCC 58053 laccase

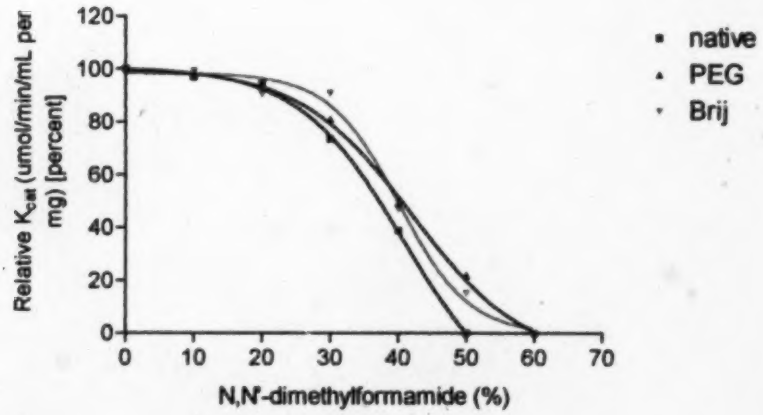


Figure 39: Effect of N,N'-dimethylformamide on K_{cat} for *P. ostreatus* UAMH 7988 laccase

3.3.3 Application of Modified Enzymes for PAH Oxidation

The modified enzymes from each strain of *P. ostreatus* (ATCC 58053 and UAMH 7988) were then utilized for the *in vitro* oxidation of several PAHs. The disappearance of the parent PAHs was followed and could be described by first-order kinetics; the data was fitted to the first-order rate equation and the rate constant for each reaction was determined. The rate constant for each of the reactions followed can be found in Table 15 for the *P. ostreatus* ATCC 58053 laccases and in Table 16 for the *P. ostreatus* UAMH 7988 laccases.

In Table 15, it can be seen that the PEG and Brij modified forms of the laccase significantly increase the rate constant (and thus decrease the half-life of the followed substrate) for 9-methylanthracene, 2-methylanthracene and anthracene. Poor oxidation is seen with all forms of the laccase for fluoranthrene and chrysene, while no significant removal of phenanthrene could be detected.

As seen in Table 16, it is apparent that the modification of laccase from *P. ostreatus* UAMH 7988 was successful in increasing the rate of reaction with 9-methylanthracene and 2-methylanthracene. Anthracene itself is oxidized, but the modifications do not significantly increase the rate of reaction. Chrysene is poorly oxidized while phenanthrene is again untouched.

Table 15: Rates of reaction of *P. ostreatus* ATCC 58053 laccases (unmodified and modified) with different PAHs

PAH	[illegible] eV	Native Rate k (hr⁻¹)	PEG Rate k (hr⁻¹)	Bio Rate k (hr⁻¹)
9-methylanthracene	7.23	10.23 ± 2.01	18.62 ± 2.84	22.59 ± 4.42
2-methylanthracene	7.42	6.85 ± 3.77	13.21 ± 1.94	14.59 ± 3.36
Anthracene	7.55	7.48 ± 2.02	15.37 ± 3.04	16.22 ± 1.89
Fluoranthrene	7.76	1.06 ± 3.95	2.94 ± 2.12	1.75 ± 0.88
Chrysene	7.80	0.052 ± 0.084	0.0021 ± 0.065	0.033 ± 0.072
Phenanthrene	8.03	NR	NR	NR

NR: no reaction

Table 16: Rates of reaction of *P. ostreatus* UAMH 7988 laccases (unmodified and modified) with different PAHs

PAH	[illegible] eV	Native Rate k (hr⁻¹)	PEG Rate k (hr⁻¹)	Bio Rate k (hr⁻¹)
9-methylanthracene	7.23	15.32 ± 4.65	24.32 ± 3.44	37.84 ± 5.21
2-methylanthracene	7.42	12.78 ± 2.65	26.63 ± 1.94	44.41 ± 8.36
Anthracene	7.55	5.21 ± 3.17	7.66 ± 6.75	4.92 ± 2.57
Fluoranthrene	7.76	2.22 ± 3.00	0.12 ± 1.78	4.55 ± 2.15
Chrysene	7.80	0.032 ± 0.014	0.085 ± 0.14	0.014 ± 0.059
Phenanthrene	8.03	NR	NR	NR

NR: no reaction

4 Discussion

As in the results section of this thesis, discussion of the results is divided into three subsections. The sections again follow the chronological order of the work performed. Strain screening and production are examined as a unit, followed by a separate section dealing with purification and characterization of the laccases. Lastly, the chemical modification of the laccases and their utility in oxidizing PAHs in organic solvent systems are considered.

4.1 Strain Screening and Enzyme Production

As with results obtained by other researchers, the screening methods employed in this report were successful in identifying high laccase producing strains in a relatively inexpensive and efficient manner. Although the precise mechanisms of colour loss or formation are unclear, the oxidation of Remazol Brilliant Blue R (RBBR) and tannic acid provided an easily visualized measure of secreted extracellular oxidative ability. While the use of tannic acid oxidation as a screening method is relatively novel, similar correlations using dye decolourization have been reported. Gold *et al.* (1988) demonstrated a good correlation between Poly R-481 decolourization and lignin degradation by *Phanerochaete chrysosporium*. Similarly, Chet *et al.* (1985) demonstrated a fit again between Poly B-411 decolourization and lignin degradation, this time with 8 different white rot fungi. As the isolates that indicated high oxidative ability with both of the substrate were *P. ostreatus* strains, further work continued with the most promising members of this group. Other researchers have also demonstrated that strains of *P. ostreatus* are capable of the production of significant amounts of extracellular oxidative enzymes (de Jong *et al.*, 1992; Esposito *et al.*, 1991; Pelaez *et al.*, 1995) and that this ability appears to be a characteristic of this genus and species of white rot

fungus. An important caveat to the screening methods used is that they are not specific for a particular type of extracellular oxidative enzyme: one could in fact be reading positive responses that are a result of lignin peroxidase or manganese peroxidase activity. While the two strains studied closely in this work did not produce either of these enzymes in liquid culture conditions, it is conceivable that the positive results seen with the other strains tested could have been a result of the activity of either of these enzymes. This possibility is heightened by the fact that some of the strains which produced promising results with the agar screening methods failed to produce significant amounts of laccase in liquid media. However, all strains producing significant amounts of laccase in liquid culture gave strongly positive results with the screening results used here.

While the agar based screening techniques provided valuable information about the general extracellular oxidative ability of the strains tested, further specific information about laccase production was required and was gleaned from liquid media experiments. These experiments utilized bran based media, which was previously shown to support superior laccase and other lignin degrading enzyme production in our laboratory (Pickard *et al.*, 1999) and by other research groups (references contained in Table 6). This observation was borne out by the fact that defined media failed to support significant laccase production with all strains tested whereas considerable but variable improvements were seen when bran based media was used.

Coincident with the study of bran media, the use of inducers to increase laccase production was also examined, using *P. ostreatus* UAMH 7988. Interestingly, syringaldehyde failed to produce an increase in laccase titer while two compounds with closely related structure, 2,6-xylidine and veratryl alcohol, which are reminiscent of lignin monomers, significantly increased titer. Success with xylidine as an inducer is not unique (Bollag and

Leonowicz, 1984; Fahraeus and Reinhammar, 1967; Yaver *et al.*, 1996) and induction of laccase levels of up to nine fold have been noted with *Pycnoporus cinnabarinus* with a similar delayed addition as that used in this study (Eggert *et al.*, 1996). However, the range of induction efficiencies varies considerably with different white rot fungi. In this study, induction efficiencies also varied but xylidine increased laccase titer by 1.2 fold and 2.7 fold for *P. ostreatus* ATCC 58053 and UAMH 7988 respectively when added at a concentration of 2 mM. Veratryl alcohol was also utilized as an inducer, and its use resulted in a 1.8 fold increase in laccase production in *P. ostreaus* UAMH 7988.

A significant portion of time was devoted to finding the optimal production protocol for laccase using the two strains selected for further study, *P. ostreatus* ATCC 58053 and UAMH 7988. While the UAMH 7988 strain was relatively uncharacterized before deposition, the ATCC 58053 strain was noted for laccase production ability by Platt *et al.* (1984) in a study of several *P. ostreatus* strains before its deposition. Several physical parameters for media composition and incubation environment were studied and optimized for laccase production; the final protocol was utilized for both ATCC 58053 and UAMH 7988 and was highly effective in both cases.

The growth curves for both of the studied fungi (Figure 17) demonstrate a pattern of enzyme secretion that is typical of those produced as a part of secondary metabolism in fungi. Production of such enzymes, like laccase, that are primarily utilized for energy acquisition, does not take place in rich media with easily accessible energy sources. Reducing sugar levels in each case are seen to fall prior to the initial increase in laccase production. The exhaustion of these easily utilized energy sources could play a role in triggering the production of this enzyme, in addition to the presence of lignin breakdown products (as evidenced by the success of the previously discussed lignin-monomer like inducers).

One of the more difficult aspects of the work at this point included scale-up of the protocol, which was initially designed only using small-scale shake flask data. However, a trial and error approach with several fermenter designs (as outlined in Results) eventually produced a final successful protocol used for overall production. While the protocol designed was very effective at 8 L of working volume, the prospects for further scale-up appear somewhat limited. This is because the highly viscous bran medium requires significant energy input via the impeller to maintain suspension and keep mass transfer optimal between the bran and the pellets in media. Such viscosity concerns could pose a significant problem with larger fermentations. Also, both of the utilized strains of *P. ostreatus* (ATCC 58053 and UAMH 7988) produced significant amounts of dark pigment during growth on bran media, and much of the purification protocol is dedicated to the removal of these compounds. Such pigment production is not seen with growth in defined media by these strains, and appears to be a characteristic of growth in bran media. One possible source of these heterogeneous coloured compounds could be the free-radical based polymerization of aromatic breakdown products of lignin in the medium. Ironically, laccase-based oxidation of aromatic substituents in the medium would convert them to free radicals capable of polymerizing with like compounds; the action of the enzyme itself would be confounding purification. However, the production of laccase in bran based media with optimal conditions is drastically higher for all tested *P. ostreatus* strains than in defined media. Few researchers have performed such an optimization using several factors. A difficulty arises in comparing production data across several research groups as the definition of an enzyme unit and substrate used can vary considerably. However, with the limited number of cases where researchers have chosen international units with ABTS (as was used in this study) it is clear that the production values obtained here are very high in comparison. Most

researchers who have used defined media for laccase production have used inducers (typically xylidine) to increase production. Pointing *et al.* (2000) utilized xylidine induction with *Pycnoporus sanguineus* to produce maximum laccase levels of 1.4 U/mL. Higher values have been obtained by other researchers using different organisms. Bourbonnais *et al.* (1995) utilized xylidine induction to produce 10 U/mL of laccase from *Trametes versicolor*; Eggert *et al.* (1996) produced laccase at 9.6 U/mL with *Pycnoporus cinnabarinus*. Values for laccase production without induction but in bran based media are typically higher than in defined media alone but lower than those seen with xylidine induction. Fukushima and Kirk (1995) cultivated *Ceriporiopsis subvermispora* on wheat bran media and observed laccase production of 0.8 U/mL. In one of the few studies utilizing *Pleurotus ostreatus*, Ardon *et al.* (1998) utilized cotton stalk extract to produce laccase at 0.95 U/mL. The values obtained in this study of 15.6 U/mL and 4.3 U/mL with *P. ostreatus* ATCC 58053 and UAMH 7988 laccase are indicative of relatively high levels of laccase production from white rot fungi. Production by recombinant means has been reported between 10 and 20 mg/L by a number of different researchers (Gianfreda *et al.*, 1999). Using the values obtained for specific activity in this study, laccase from *P. ostreatus* UAMH 7988 and ATCC 58053 was produced at 45 mg/L and 255 mg/L respectively. Clearly, time was well spent in this study manipulating production conditions with the native fungi for optimal secretion of laccase given the significant gravimetric quantities produced. However, if similar values for production could be achieved with recombinant production, such a system would be ideal given the relatively easier purification possible from defined media.

4.2 Purification and Characterization

The protocol designed for purification in this study was successful in producing several milligrams of pure laccase. The yield and percent recovery achieved in this experiment (Tables 9 and 10) are similar to those obtained by other researchers. In general, the purification protocols utilized by other researchers have a similar number of steps. A few protocols have fewer steps, probably a function of the fact that most production in these cases comes from growth in defined media, with fewer contaminants to remove (but lower overall enzyme levels) compared to fermentation with complex bran sources as was performed in this experiment. Most researchers utilize anion and/or size exclusion chromatography and typically only need two column purification steps. Dedeyan *et al.* (2000) purified a laccase from *Maramius quercophilus* with a three step chromatographic procedure to a final yield of 31 % and a purification factor of 2.4 fold; these values are at the low end of the range reported. Conversely, Ridgway *et al.* (1999) utilized similar techniques to purify laccase from apple leaf 49 fold with a yield of 75 %; these are the highest values found for these parameters. The values obtained in this report are representative of those obtained by a number of other researchers despite the confounding influence of the heterogeneous pigment produced by both the fungi studied.

No isoenzymes were demonstrated for either enzyme during purification in this study; the conditions chosen for production appear to support the production of only one dominant form of laccase. This is somewhat unusual, but not completely unknown. In isolating laccase from *P. ostreatus*, Palmieri *et al.* (1993) described the production of 3 isoenzymes (with growth in potato dextrose broth); two were found in trace quantities while the third was the most abundantly secreted by far. This third enzyme was later further characterized by Giardina *et al.* (1996) and described as the product of a sequenced *pox2*

gene. However, other researchers have reported the production of single forms of laccase from white rot fungi. Kovoljova *et al.* (1999) described a single form of laccase from *Coriolus zonatus* as did Heinzkill *et al.* (1998) with *Panaeolus sphinctrinus* and *Panaeolus papilionaceus.*

The polyacrylamide gel in Figure 25 demonstrates some microhetereogeneity in the bands seen with total protein stain for both of the isolated laccases. This is most likely a consequence of the fact that these proteins are located extracellularly and as such are exposed to significant proteolytic and glycosidic activities (Thurston, 1994). Thus nicking of the polypeptide or carbohydrate chains of the protein can lead to minor differences in migration through the gel. In addition, later data from N-terminal sequencing and other characteristics indicated that only a single enzyme species was present. The A_{280} to A_{610} ratios for these laccase from *P. ostreatus* ATCC 58053 and UAMH 7988 were 11.9 and 10.9 respectively; this figure relates the absolute amount of protein present to type 1 copper. Typical ratios of this type for fungal laccases are between 10 and 20. Isoenzymes from *Trametes versicolor* have ratios of approximately 18 (Bourbonnais *et al.*, 1995), those from *Ceriporiopsis subvermispora* are 20 (Fukushima and Kirk, 1995) and values for *Pleurotus eryngii* isoenzymes are around 15 (Munoz *et al.*, 1997). Lower values for a given enzyme indicate greater purity to a characteristic minimum value indicating maximum purity.

Interestingly, while the A_{280} to A_{610} ratios were similar for both the enzymes purified in this study, they had dissimilar specific activities. The specific activity for laccase from *P. ostreatus* UAMH 7988 was 1.6 fold greater than that of the laccase from ATCC 58053 (95 U/mg compared with 61 U/mg). Therefore, while the complement of reactive copper is most likely quite similar in both cases, other differences exist that impart the laccase from UAMH 7988 with a greater catalytic efficiency (for the substrate ABTS) than the laccase from ATCC 58053. Such differences could include different active site conformation or

dynamic stability. The values obtained for specific activity of these laccases are similar to those seen by other researchers for laccases from different organisms. The LI and LII isoenzymes from *T. versicolor* have specific activities of 160 U/mg and 45 U/mg respectively (Bourbonnais *et al.*, 1995) and the L1 and L2 isoenzymes from *C. subvermispora* have specific activities of 310 U/mg and 220 U/mg respectively (Fukushima and Kirk, 1995).

The characterization of the isolated laccases provided useful information about their activities and also provided further clues about their physical nature. Another of the major differences seen with the two laccases studied was their difference in temperature optima and glycosylation. The two subjects may in fact be linked; the greater temperature optima (60 °C vs. 40 °C) exhibited by laccase from *P. ostreatus* ATCC 58053 over UAMH 7988 may in fact be a function of the greater total carbohydrate content seen (18.4 % and 15.2 % for ATCC 58053 and UAMH 7988 respectively). The temperature optima reported here are for catalysis over the initial 15 s of the reaction; the substrate solution was pre-incubated to the desired temperature in the spectrophotometer and absorbance changes were followed immediately after enzyme addition and agitation. All reactions were linear over the time followed and demonstrate activity before significant denaturation could take place. Additional glycosylation has been hypothesized as a source of stability for extracellular enzymes by several researchers. Recently, Rudd *et al.* (1994) demonstrated that the degree of glycosylation can directly affect both the stability and activity of enzyme preparations. More specifically, Yoshitake *et al.* (1993) demonstrated that the carbohydrate moiety of *Trametes versicolor* conferred resistance to elevated temperatures and proteolytic attack. The degree of glycosylation of these enzymes is very similar to that obtained by other researchers for several laccases, including 10 % for *Coriolus zonatus* and 15 % and 10 % for the L1 and L2 laccases from *Ceriporiopsis subvermispora* (Fukushima and Kirk, 1995; Koroljova *et al.*, 1999).

However, the different degrees of glycosylation of the two laccases in this study does not appear to confer a significant difference in longer term temperature stability as both appear to be rapidly denatured at 65 °C.

Fungal laccases generally display acidic pH optima and this is the case with the laccases from *P. ostreatus* ATCC 58053 and UAMH 7988. Most fungal laccases display optima in the range of 3 to 5 (Chefetz *et al.*, 1998), and both *P. ostreatus* ATCC 58053 and UAMH 7988 fall within this range with optima of 3.0 using ABTS as the substrate. Palmieri *et al.* (1993) have demonstrate that the pox2 laccase isolated from *Pleurotus ostreatus* (Florida) had a similar pH optima at 3.0 for ABTS as well. Clearly, pH optima differs depending on the substrate utilized, but all of the substrates tested in this study were oxidized more efficiently at acidic pH optima. The differences seen in pH optima with different substrates are usually a function of their differential protonation. The resultant change in ionization of the substrate can drastically affect the catalysis mediated by laccase (or other enzymes). However, ABTS oxidation is a good measure when determining pH optimum as its oxidation is not pH dependent with laccase in the range of pH 2 to 11 (Majcherczyk *et al.*, 1998).

The molecular weights of the two laccases examined in this study were estimated using SDS-PAGE; however, more accurate values were obtained using MALDI-TOF analysis. The values obtained of 55 526 Da for *P. ostreatus* ATCC 58053 and 55 349 Da for *P. ostreatus* UAMH 7988 are essentially identical; the higher value obtained for ATCC 58053 may be a function of the greater degree of glycosylation observed. These values are slightly smaller than the 60 kDa predicted for molecular weight by SDS-PAGE. This slight downward trend from SDS-PAGE to MALDI-TOF analysis was also observed by Palmeiri *et al.* (1997) in the isolation and characterization of a unique laccase from *P. ostreatus*.

One of the most distinctive features of an enzyme is its N-terminal sequence of amino acids; once these sequences were determined, it was seen that in addition to being identical to each other, they were also identical to that determined for the pox2 laccase isolated earlier by Palmieri *et al.* (1993) and sequenced by Giardina *et al.* (1996). It is highly probable that the overall sequences also share significant homology. This assumption was utilized during chemical modification to estimate the overall number of free amino groups (lysines) available for chemical attachment. Sequence information from pox2 indicates that there are probably 6 lysine groups, which if accessible on the outside of the protein, could be modified chemically. Since 34 % and 45 % of the free amino groups on *P. ostreatus* ATCC 58053 and UAMH 7988 were modified respectively, this would translate into between 2 and 3 lysine residues being modified per laccase moiety.

4.3 Chemical Modification and Application

Chemical modification with monofunctional polymers has been utilized by several researchers to modulate enzyme activity in organic solvents. Most work in this area has centered on reactions that would directly benefit from action in an organic milieu, with enzymes such as lipase, chymotrypsin, subtilisin and catalase. Oxidative enzymes have also been examined as well, with horseradish peroxidase, cytochrome c and lignin peroxidase receiving attention. As mentioned in the introduction, the effects of organic solvents on enzyme activity are well documented, and losses in activity have been noted for all of the enzymes listed above. However, relatively few (Khmelnitsky *et al.*, 1991; van Erp *et al.*, 1991; Vazquez-Duhalt *et al.*, 1993) have analyzed maximum catalytic ability over a range of solvent concentrations as was performed in this study. The threshold dependent behaviour of laccase inhibition observed in this study with each of the three solvents tested (acetonitrile,

dimethylsulfoxide and N,N'-dimethylformamide) was very similar to the effects seen by these other researchers. The EC_{50} values for each of the two native laccases in this study are reached at ca. 40 % solvent in all cases, except for the acetonitrile with native ATCC 58053 laccase (29.3%). Values obtained by other researchers include an EC_{50} at 32% methanol with native α-chymotrypsin (Khmelnitsky *et al.*, 1991), and EC_{50} values at 73 % methanol and 68 % 2-propanol using cytochrome c (Vazquez-Duhalt *et al.*, 1993).

Most workers in this area have focussed on the increases in activity (although not always by measuring a full panel of V_{max} and K_m values) associated with modification. Several have used polyethylene glycol (PEG) to modify enzymes, but modification with Brij35 is still a relative novelty. Heriaz *et al.* (1999) demonstrated an increased stability of lipase from *Candida rugosa* in isooctane after covalent modification with PEG; similarly, activity of lipases from several sources at specific organic solvent concentrations has been noted to increase after similar modifications (Koops *et al.*, 1999; Secundo *et al.*, 1999). Vazquez-Duhalt *et al.* (1993) demonstrated that modification of horse heart cytochrome c with PEG increased K_{cat} four fold in 90 % tetrahydrofuran and more than doubled stability in the same system. In an extensive study with α-chymotrypsin, Khmelnitsky *et al.* (1991) increased the volumetric % of solvent EC_{50} was seen at by an average of 22% using 8 different water:cosolvent systems. Modification of lignin peroxidase with PEG has also been noted to increase activity to twice that of the native enzyme in 10% acetonitrile (Wang *et al.*, 1999). Brij35 modification of catalase has been used to improve activity in 1,1,1-trichloroethane by 200 fold over the native form of the enzyme (Jene *et al.*, 1997).

Each of the solvents analyzed in this study had a similar threshold effect on reducing activity of the laccases tested. The modifications performed with PEG and Brij35 had nearly identical effects with each laccase, however, different effects on maximal activity were seen

with each solvent. The increases in EC_{50} reported here are similar to those outlined above but are smaller in magnitude than those seen in those most directly comparable studies of Khmelnitsky *et al.* (1991) and Vazquez-Duhalt *et al.* (1993). This raises an important point; the comparison of data from several research groups interested in enzyme activity in general and in organic solvents specifically is difficult because of the use of several methods for both determining this activity and reporting the data. A more systematic and unified approach could help to rapidly expand our knowledge in this area. The results of this study indicate that the modification of laccase with PEG and Brij35 to the extent reported here are most successful in maintaining activity in acetonitrile, followed by dimethylsulfoxide, and appear to have little or no effect in preventing denaturation in N,N'-dimethylformamide. Table 17 summarizes the EC_{50} values obtained in this experiment for each solvent and enzyme tested. While significant differences exist in the activity of each enzyme in the different solvents tested, no correlation between the physical parameters of the solvents tested and these differences could be found.

It is apparent that a similar trend to that seen with maximal activity can be uncovered in analyzing the data describing the half-lives of the different forms of the native and modified laccases tested in this study. Incubation in acetonitrile resulted in the briefest half-lives for both ATCC 58053 and UAMH 7988 laccase (2.0 and 1.3 min respectively). However, the most dramatic increases in half-life were seen with respect to acetonitrile and were similar with both laccases; modification with PEG roughly tripled half-life while modification with Brij35 increased half-life by approximately a factor of seven. It appears that modification with Brij35 has a greater effect in increasing stability in acetonitrile, whereas PEG modification had a marginally greater effect in increasing initial activity in acetonitrile. Similarly to the data for initial activity, modification of these enzymes had less

Table 17: Percentage of solvent resulting in 50% activity (EC_{50}) for native and modified laccases from *P. ostreatus* ATCC 58053 and UAMH 7988

	P. ostreatus ATCC 58053			*P. ostreatus* UAMH 7988		
	Native	PEG	Brij35	Native	PEG	Brij35
CH_3CN	29.3	43.7	34.4	39.3	47.6	44.5
DMSO	45.0	49.6	51.1	44.9	50.0	47.7
DMF	41.1	44.3	41.8	40.7	42.0	40.1

effect on stability in dimethylsulfoxide then it did with acetonitrile. Both PEG and Brij35 modifications produced an increase in half-lives of approximately two-and-a-half fold over the native enzyme for native ATCC 58053; no significant effect was noted with UAMH 7988 in this system. Again similarly to results obtained for initial activity, no significant effects on half-life could be determined for modification in N-N'-dimethylformamide. Interestingly, the modified and native laccases from *P. ostreatus* ATCC 58053 demonstrate a greater half-life in each of the solvents tested than the laccase from UAMH 7988.

Modification of the laccases in this study did not affect overall substrate range (Table 13). However, about a tenfold decrease in K_m was noted after each modification for both ATCC 58053 and UAMH 7988 laccases with 2,6-dimethoxyphenol as the substrate. Both modifications of UAMH 7988 also produced an approximate tenfold increase in K_m when examining the oxidation of 4-aminophenol. It is conceivable that some sort of slight conformational change could be responsible for these small differences, especially given the fact that the typical absorbance peak for the type 1 copper at 610 nm disappears after modification in all cases. However, it is clear that the catalytic range and activity of these enzymes were not dramatically altered by modification and are in fact representative of values found for other laccases as well; Table 18 illustrates some comparable values obtained by other researchers.

If the assumption (supported by the K_m data in Table 13) is made that substrate range and specificity were not drastically affected by the modification of the tested laccases, another explanation is required to rationalized some of the data on PAH oxidation in Tables 15 and 16. The increases in stability and activity outlined above support the inference that it is augmentation of these factors that led to a greater rate of oxidation for the PAHs with an ionization potential of $\leq$ 7.55 eV for ATCC 58053 laccase and $\leq$ 7.42 for UAMH 7988

Table 18: K_m values for representative substrates with laccases isolated from several different sources.

Compound	Source of Laccase	K_m (mM)
ABTS	*Chaetomium thermophilium*	0.19
	Ceriporiopsis subvermispora	0.03
	Pleurotus ostreatus (florida)	0.28
	Trametes villosa	0.058
	Pycnoporus cinnabarinus	0.018
	***P. ostreatus* ATCC 58053**	**0.19**
	***P. ostreatus* UAMH 7988**	**0.048**
Syringaldazine	*Chaetomium thermophilium*	0.034
	Pleurotus ostreatus (florida)	0.015
	Trametes villosa	0.004
	Pycnoporus cinnabarinus	0.004
	Myceliophthora thermophila	0.01
2,6-dimethoxyphenol	*Chaetomium thermophilium*	0.096
	Ceriporiopsis subvermispora	2.9
	Pleurotus eryngii	1.4
	***P. ostreatus* ATCC 58053**	**1.26**
	***P. ostreatus* UAMH 7988**	**2.22**
Guaiacol	*Chaetomium thermophilium*	0.40
	Ceriporiopsis subvermispora	1.6
	Pleurotus eryngii	2.2
	***P. ostreatus* ATCC 58053**	**3.32**
	***P. ostreatus* UAMH 7988**	**5.16**

Values obtained from Gianfreda *et al.* (1999) except for bolded values which were determined in this study.

laccase. Acetonitrile was chosen as a solvent for the *in vitro* oxidation of PAHs because of its ease of use in the HPLC system utilized and the fact that the increases in activity and stability over the native enzyme were the most dramatic when this solvent was used. Modification with PEG and Brij35 appear to have a very similar effect in increasing the rate of oxidation of PAHs with a given amount of ATCC 58053 laccase. Table 15 demonstrates that 9-methylanthracene, 2-methylanthracene, and anthracene are all oxidized at significantly greater rates with the modified forms and the increases are not significantly different from each other with each PAH tested. However, the rate of oxidation of PAHs tested with laccase from UAMH 7988 does appear to depend on the method of modification for 9-methylanthracene and 2-methylanthracene, as evidenced in Table 16. In both cases the Brij35 modified enzyme shows a significantly greater rate of reaction than the PEG modified enzyme. In contrast to the laccase from ATCC 58053, modification of the laccase from UAMH 7988 does not appear to increase the rate of oxidation of anthracene. In the cases with these two enzymes where there was a significant effect from modification, it is likely that increases in rate were due to the increases in stability and activity achieved, presumably as a result of the increased ability of the enzymes to maintain critical hydration.

An alternate hypothesis explaining the increase in oxidation of PAHs after modification is also possible. Given the amphipathic nature of the polymers used to modify laccase in this study, increases in the surface hydrophobicity of the enzymes could also have taken place (Vazquez-Duhalt *et al*, 1992). In addition to trapping water at the enzyme surface, the modifications could have played a role in increasing the rate of reaction by partitioning hydrophobic PAHs from solution to the surface of the enzyme. However, it is more likely that the increases in stability and activity in organic solvents played a greater role in determining the overall rate of PAH oxidation.

The threshold effect observed here in terms of substrate range of PAHs is similar to that seen by other researchers using laccase to oxidize PAHs. The PAHs with the highest ionization potentials in this study, fluoranthrene, chrysene and phenanthrene, were not oxidized to any significant extent; presumably, they are resistant to oxidation because of their relatively higher ionization potential. Other steric factors could also potentially be playing a role; these PAHs could fit the active site more poorly than anthracene and its derivatives oxidized in this study. However, given the relatively non-specific nature of the active site of laccase (as evidenced by its wide substrate range) a more likely explanation is that the ionization potential of these compounds was too high for laccase to facilitate the removal of an electron. Both of the laccases in this study appear to be similarly limited in their inability to oxidize PAHs with ionization potentials above about 7.5 eV. The results obtained here are similar to those seen in a study by Collins *et al.* (1996) where ionization potential appeared to play a role in the oxidation of PAHs by laccase from *Trametes versicolor*. These researchers demonstrated that this laccase was similarly limited to oxidation of PAHs with an ionization potential ≤ 7.45 eV. Later studies by Majcherczyk *et al.* (1998) and Johannes and Majcherczyk (1996) demonstrated that the range of PAHs oxidized by *Trametes versicolor* laccase may be increased on the inclusion of mediating substances in the reaction mixture, such as ABTS and 1-HBT. However, the rates of reaction in this study for PAHs with lower ionization potentials (i.e. anthracene) were greater than those with higher ionization potentials (i.e. phenanthrene). This same general trend was noted by Pickard *et al.* (1999) using laccase from *Coriolopsis gallica*. The laccases in this study appear similarly limited in their substrate range, however, the modifications performed significantly increase oxidation rate of those PAHs with low enough ionization potentials.

4.4 Concluding Remarks

In summary, the goals of this project were to produce large amounts of laccase and increase the utility of the purified enzyme in PAH oxidation in an organic solvent system. Through several steps, the overall goals of this work were reached. Two strains of *P. ostreatus* (ATCC 58053 and UAMH 7988) were chosen for laccase production based on promising results from the screening of several organisms. The screening techniques used were of high utility and could be used in future studies of this type. Optimization of production conditions for these strains resulted in the production of large amounts of laccase, at values rivaling other methods of production, including those employing recombinant techniques. The purification of these enzymes was typical and produced preparations of high purity; no isoenzymes were isolated. Characterization of these enzymes indicated that they were similar to fungal laccases in general and more closely to a previously characterized laccase from *Pleurotus ostreatus*. Information gleaned from characterization was useful in the chemical modification of these enzymes with the amphipathic polymers PEG and Brij35. Both of the chemical modifications were successful in increasing the activity and stability of the laccases in acetonitrile and dimethylsulfoxide but not N,N'-dimethylformamide. The modified enzymes displayed greater rates of PAH oxidation in acetonitrile systems than their native counterparts. PAH oxidation by these laccases was similar to that seen by other researchers as it was limited by the ionization potential of the PAH. These modified laccases could be useful in further optimized PAH oxidation systems, as well as in other applications requiring oxidation in organic solvent systems. Further, modification with PEG and Brij35 may be useful for other enzymes whose activities are required in similar systems.

5 Future Study

5.1 Utilization of Laccase

Laccase is currently used in several industries, including pulp and paper. However, the number of uses that are possible for this enzyme could be expanded by efficient catalysis in organic solvents. Also, laccase has been extensively studied in small-scale experiments, however, there is a paucity of data in regards to scaling up these small pilot-scale studies. Examining the increased range of reactions possible and the meaningful incorporation of select ones into full-scale processes could be of significant economic and social benefit.

5.2 Further Characterization

As a prelude to further enhancement of activity in organic solvents, it would be beneficial to determine more information about the structure of the enzyme. Information learned could be applied to the elucidation of important sites of hydration and how best to protect them from dehydration. The crystal structure of laccase has yet to be determined; this alone would be an important step.

5.3 Modification

Many techniques for enzyme modification exist and could be of benefit for increasing the stability and activity of laccase in organic solvent. The modifications in this study were targeted only at the accessible amino groups of lysines, a very specific subset of the overall structure of the protein. Similar modifications at other groups could be attempted: techniques for addition to phenolic and carboxy groups are well described in the

literature. Enzyme modifications that yield soluble products are useful in many cases. However, despite their advantages, soluble enzymes can be difficult to recover during industrial processes. For this reason, it may be interesting to see if the modifications performed by Ito *et al.* (1999) where enzyme solubility could be manipulated by irradiation with different wavelengths of light could be applied to laccase. Laccase modifications which yeild insoluble forms of the enzyme may also prove to be of greater utility and should be examined. The creation of laccase cross-linked crystals should be examined. While several researchers have studied the adsorption of laccase to supports, further work here could also provide useful answers.

5.4 Mediators

Mediator action is well known to expand the substrate range and rate of oxidation of laccase. While several mediators are known, it would seem prudent to expand the number of compounds tested given the dramatic effect that they can have on catalysis. In addition, the interaction of laccase and mediators in organic solvents has yet to be well described.

5.5 Reaction Products

The reaction products formed as a result of laccase action on PAHs in this experiment and several like it still have to be characterized. Oxidation is clearly taking place and some of the limited information that is available supports the assumption that a variety of quinones are formed. However, the situation needs to be clarified; one possible discovery could be the ring cleavage of certain PAHs mediated by laccase. Further, the relationship between mineralization of PAHs and exoenzyme expression by white rot fungi needs to be examined further.

5.6 Laccase Production

From a physiological standpoint alone, the mechanisms governing increased laccase production in the presence of lignin or its subunits would be interesting to examine. This area appears to be devoid of study and could provide a wealth of information about fungi and secondary metabolism in general as well. The large increases observed in this study and others demonstrate that the mechanisms governing production are capable of significant and rapid shifts in enzyme output.

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7 Appendix

7.1 Enzyme Kinetics

Values for the kinetic parameters of enzyme reactions (K_m and V_{max}) were calculated by fitting data directly into the Michaelis-Menten equation:

$$V = \frac{V_{max}[S]}{[S] + K_m}$$ where V= the reaction rate and S= the substrate concentration

and performing non-linear regression using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA.

Kinetic data was initially plotted with Lineweaver-Burke and Eadie-Hofstee plots; data sets yielded straight lines after these reciprocal transformations. However, more accurate and precise values were obtained by fitting data directly to the Michaelis-Menten equation, thus avoiding errors typically introduced by linear transformation.

7.2 Calculation of EC_{50}

Threshold effects observed in nature (i.e. cell depolarization events) have been described using the Boltzmann equation:

$$Y = \frac{\text{lowest value} + (\text{highest value} - \text{lowest value})}{1 + \exp[(EC_{50} - X)/\text{Slope}]}$$

Fitting the enzyme denaturation data to this curve yielded extremely reproducible curves that fitted the data well. In addition, this equation was used to calculate EC_{50}, the concentration of solvent at which 1/2 of maximal enzyme activity is observed. GraphPad Prism version

3.00 for Windows, GraphPad Software, San Diego California USA, was used to fit the data to the Boltzmann equation.

7.3 PAH Oxidation Rates

The rate of oxidation of PAHs typically fit a curve well described by first-order decay, indicating that the rate of oxidation was proportional to the amount of PAH remaining in the reaction mixture. Time course data was fitted to the equation:

$Y = Y_{\circ} \exp(-kx)$ where Y = concentration at time x; Y_0 = intial concentration

and in this report, the rate constant, or k, is reported, with a greater rate constant indicating a quicker time of removal; half-life of the PAH in this case can be found by:

$T_{1/2} = 0.693/k$

The data was fitted directly to the first-order decay equation and non-linear regression was performed using using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA.